ANNUAL REPORT
OF
PROGRAM ACTIVITIES

NATIONAL INSTITUTE OF
ALLERGY AND INFECTIOUS DISEASES

FISCAL YEAR 1972

U.S. DEPARTMENT OF HEALTH, EDUCATION, AND WELFARE
PUBLIC HEALTH SERVICE NATIONAL INSTITUTES OF HEALTH

ANNUAL REPORT

OF

PROGRAM ACTIVITIES

U.S. NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

Fiscal Year 1972

DIRECTOR
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EXTRAMURAL
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ANNUAL REPORT OF PROGRAM ACTIVITIES
NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES
JULY 1, 1971 THROUGH JUNE 30, 1972

The National Institute of Allergy and Infectious Diseases conducts and supports a broad program of research directed at helping medical science find improved ways of diagnosing, treating, and preventing allergic conditions and infectious diseases. In carrying out its mission the Institute continues its role of training future medical research workers.

The senior staff of the Institute and the basic organization continue the same as last year. A significant change was the incorporation of research contract management into the Office of the Director, NIAID, effective December 23, 1971. Authority for awarding contracts was delegated to the Institute Director, and contract officers were appointed with the approval of the Department of Health, Education, and Welfare. To meet this new responsibility, the Institute established a Contract Management Branch of which Mr. Merle Callahan is Chief. He has responsibility for soliciting, review and negotiation of research contracts related to Institute programs. Early indications are that this arrangement provides better and more efficient coordination of the process of contracting than the former arrangement.

A major loss to the intramural program was the disestablishment of the Middle America Research Unit, an intramural research laboratory of the Institute located in the Panama Canal Zone. The immediate reason for this action was the requirement to reduce Institute personnel significantly coupled with the expectation of significant changes in the senior research personnel at MARU and a changed emphasis of research direction in Institute programs. The Middle America Research Unit, established in 1957, contributed greatly to our knowledge of the arboviruses in Central and South America. The studies on Bolivian hemorrhagic fever alone are classic examples of laboratory and field investigations which led directly to a practical means of control of the highly fatal human infection. In addition to the influence that MARU has had in acquisition of new knowledge MARU served as a training ground for numerous young investigators who have continued their interest in research and in the arboviruses.

This year, the Research Information Section has sought to emphasize the allergy and immunology aspects of the Institute's mission. A 72-page publication, "Clinical Immunology, New Approaches to Treatment Through Management of the Immune System" was produced as a follow-up to a successful Science Writers' Seminar held last year in Atlantic City. It is being distributed to science writers and to interested physicians and scientists. Our first publication on allergy research written for the general public, called "Allergy Research, An Introduction," is in press.

Together with the Institute's Transplantation and Immunology Branch and the National Institutes of Health/American College of Surgeons (NIH/ACS) Organ Transplant Registry, the Section is publishing a fact book on U. S. kidney

transplants. This book will contain a brief history of organ transplantation and selected tables based on information from the Registry's computer operation. It will be useful to transplant surgeons, immunologists and science writers.

The Section co-sponsored a Roundtable for Science Writers on Allergy with the American Academy of Allergy as a prelude to the Academy's annual meeting. Conference telephones were set up at the University of California Medical Center campus in San Francisco and at the Institute's Conference Room in Bethesda. The experimental 3-hour coast-to-coast hook-up by FTS lines provided for 2-way conversations between the scientists in San Francisco and science writers in San Francisco and Bethesda.

The series of Infectious Diseases Research Fact Sheets has been an invaluable time-saver to respond to both press and public inquiries. There are now 18 fact sheets. New fact sheets were written on toxoplasmosis, schistosomiasis, leprosy and venereal diseases, and seven of the earlier fact sheets were updated during the year.

The Section has produced a brochure on the Institute's contracts program to be distributed primarily to current and prospective contractors. A similar publication on the Institute's grants and awards programs -- including Extramural Programs as well as the grants aspect of the Institute's Geographic Medicine Branch -- is in press. For the second year, a short-run publication, "Highlights from the Annual Report of NIAID," has been produced to explain current research activities and accomplishments of the Institute in layman's terms. The Section also produced a series of black-and-white and color photos of activities in the Bethesda laboratories to be used in future public information presentations.

During its second year of operation, the statistical unit in the Office of the Director (1) continued to accumulate and evaluate available data from many sources on the prevalence of allergic diseases and their medical cost and economic implications; (2) served as project officer for a contract to develop standard criteria for the diagnosis of allergic diseases; (3) developed plans and drafted specifications for a program to produce new data on allergic prevalence, and on the utilization of medical services for the diagnosis and treatment of allergic diseases; (4) prepared a paper on the problems of measuring allergic prevalence.

The distribution of new data and scientific communications between research invesigators constitute an important part of the support of biomedical research. Scientific communications that are prompt, broad, accurate and effective help to assure the efficient expenditure of research efforts and funds. The Institute has a continuing interest in exploring new and improved ways to increase scientific communications. An example exists in the trend away from large multisubject scientific conferences composed mainly of a procession of 10-15 minute papers. Growing in usefulness and popularity are the small, workshop type meetings, where, based on a few carefully prepared statements of position papers, a single subject is discussed in depth. Another example is the development of a means for rapid, frequent and informal Institute-to-scientist communications known as the Scientific Memoranda.

The main product of the Institute is new information stemming from the research funded during the year and made available to the research community, educational institutions, medical practitioners and other interested persons through publication of manuscripts in scientific journals and special reports. During FY 72 approximately 350 manuscripts and research notes were produced by the scientific staff of the Institute. An estimated 2000 were published by grantees and approximately 200 by research contractors. In addition, 120 technical or progress reports from research contracts were received by the Institute, and copies sent to the Department of Commerce "National Technical Information Service" from where they are available to scientists and the public.

A valuable monograph "The Primate Malarias" authored by members and ex-members of the Institute was published by the Government Printing Office. Institute support of this publication, which included 24 color plates, made it available at a nominal cost to scientists and students. Partial support was provided for publication in English of the Soviet book "Bloodsucking Tick (Ixodoidea) Vectors of Diseases of Man and Animals," which had been translated through a PL480 funded project. Also produced in this PL480 funded collaborative project, between the Institute and the U. S. Naval Medical Research Unit in Cairo, Egypt, were three of a four-volume "Bibliography of Ticks and Tickborne Diseases." In collaboration with the Fogarty International Center, a book "Immunization in Tuberculosis" was edited and published. The text was composed of the prepared papers and discussion of a conference with the same title.

The NIAID feasibility project for development of a mechanism for fast and informal scientific communications between research investigators progressed satisfactorily and has been expanded. Each of the "Scientific Memoranda" is devoted to a special field of research and the communications are composed of abstracts, progress reports and other informal notes on current research. Participants are the pertinent active investigators throughout the world and each must submit at least one communication per year to remain in the project. In FY 72, the "Interferon Scientific Memoranda" appeared monthly with an average of 14 communications per issue; the "Leprosy Scientific Memoranda" had an average of 26 communications on a tri-monthly distribution, and the bi-monthly "Hepatitis Scientific Memoranda" contained an average of 25 informal communications. In May 1972 two new projects were initiated in areas of special program interest to the Institute; the "Immediate Hypersensitivity Scientific Memoranda" with over 50 members and the "Neisseria Treponema Scientific Memoranda" with approximately 40 participants. These are distributed on a bi-monthly basis. Analyses of these projects reveal that the early warnings resulting from these informal communications permit investigators to make timely improvements in their experiments and aid in avoiding needless duplications, thus saving in research efforts and funds many times the cost of the "Scientific Memoranda" Project.

In the conduct or support of scientific meetings a continuation was seen in the trend to more small meetings, each devoted to indepth discussion of a specific and narrow field. Funding was provided in FY 72 for only three large conferences (i.e., 200 or more attendees), and in each case only partial support was provided by the Institute. On the other hand, 63 workshop

Working conference meetings were either conducted by NIAID or partial funding provided via grants or contracts. Attendees ranged from 12 to 120. Subjects were related mainly to the special emphasis programs of NIAID and in most cases provided a summary and evaluation of research progress. Often serving the dual purpose of advice to the Institute on program plans and scientific communications between the participating scientists, special efforts were made to publish summary reports in appropriate scientific journals. In the intramural laboratories an average of two seminars per week were conducted, many of them presented by the 25 visiting consultants and scientists who spent short periods in the Institute.

Preliminary experiments were begun in a new feasibility project to develop the use of communication satellites for scientist-to-scientist and other scientific communications.

GEOGRAPHIC MEDICINE BRANCH

1. SUMMARY

The Geographic Medicine Branch was created in the NIAID in 1968 to manage three international programs: The United States - Japan Cooperative Medical Science Program, the International Centers for Medical Research and Training (ICMRT), and the International Research Career Development Program (IRCDP). The IRCDP has since been modified and incorporated within the ICMRT Program.

I. The United States - Japan Cooperative Medical Science Program

Background

During their meeting in Washington, D.C. in January 1965, the Prime Minister of Japan and the President of the United States gave purposeful recognition to their common and urgent concern for the health and well being of all the people of Asia. A new bilateral program was proposed and named the United States - Japan Cooperative Medical Science Program (U.S.-Japan CMSP). The two heads of state agreed to undertake a greatly expanded, joint cooperative research effort in the biomedical sciences, with a specific focus upon health problems of recognized importance in Asia.

Initially, the governments of both United States and Japan appointed prominent medical advisors from their respective countries as Delegates to a joint U.S.-Japan Cooperative Medical Science Program Committee. These consultants continue to review jointly the Program's objectives, operations, and accomplishments. This Joint Committee has met annually since 1965 alternating between locations in the United States and Japan. The seventh and most recent meeting was held at the NIH, Bethesda, Maryland on July 29 and 30, 1971.

The Program is concerned with the diseases of substantial health importance in Asian countries. The diseases or categories of diseases are: 1. cholera, 2. leprosy, 3. malnutrition, 4. the parasitic diseases, schistosomiasis and filariasis, 5. tuberculosis, and 6. selected viral diseases (rabies, dengue-hemorrhagic fever, and certain arbovirus diseases).

The overall U.S.-Japan Cooperative Medical Science Program operates within a bilateral government framework. Nevertheless, it may involve scientists and facilities in third countries, and/or collaboration with international or other organizations. Joint third country activities are approved in advance by the U.S. and Japanese Delegations.

The relevant regions in Asia are not specifically defined, but are generally understood to include the Republic of Korea on the north, India and Pakistan to the west, and other adjacent nations in the broad Pacific basin.

The United States Delegation to this Committee is appointed by our Secretary of State, and Japan's Delegation by the Japanese Ministry of Foreign Affairs. The Delegations advise their respective governments on the scope, direction

and other broad aspects of the Program, and develop review procedures to assure fulfillment of the purposes for which the Program was established. Jointly the United States and Japanese Delegations have designated the six areas of research which fall within the scope of the overall Program.

In Japan the Program activities are the responsibility of the Ministry of Health and Welfare and the Ministry of Education. In the United States the operational responsibility for the Program has been delegated to the National Institutes of Health (NIH), Department of Health, Education and Welfare. The United States and Japan maintain separate secretariats.

For each of the six areas selected for study, ten-member joint Panels, consisting of five scientists from each nation, were established to delineate more specifically areas for mutual cooperation and to carry on Program activities. Briefly summarized, these activities may be grouped into the following three categories: support of research, joint scientific conferences, and exchange of persons. In addition, each Panel prepares an annual report of progress for review by the Joint American and Japanese Delegations at their annual, joint meetings. The Panel scientific meetings, as do the Delegation meetings, alternate annually between locations in the United States and Japan.

The United States and Japan each support the cost of their own scientific projects and meetings. Research by U.S. scientists is funded by the United States and that of Japanese scientists by Japan. Exchange of scientists between the two countries is encouraged as a matter of principle and is supported by official or unofficial agencies of either country. The U.S. does not fund research in Japan nor grant fellowships to Japanese scientists under this Program, nor does Japan fund U.S. research or fellowships for Americans as a component of their Program-sponsored activities.

The National Institutes of Health, based upon a specific delegation of Presidential authority under Public Law 86-610, the International Health Research Act of 1960, and on the availability of funds, provides:

- support of scientific projects
- organization, funding, and conduct of scientific meetings held in the United States
- financial support to the United States Panel members and consultants attending meetings related to the Program
- staff and operating costs of the U.S. Secretariat

The Japanese Ministries of Health and Welfare and of Education assume a similar responsibility for their counterpart activities, i.e. research projects meetings held in Japan, Japanese Panel members and consultants, and for the Japanese secretariat.

Highlights of the Past Year

CHOLERA

Important achievements of cholera research during the past year relate to a more precise delineation of how the cholera enterotoxin alters fluid movement across the intestine, further improvement in the toxoid vaccine, and a deeper understanding of the mechanism by which other bacterial pathogens cause diarrhea.

Investigations in several laboratories have clearly demonstrated that the cholera enterotoxin stimulates adenyl cyclase in the intestinal mocusal cells, and this stimulation of adenyl cyclase is most likely responsible for the intestinal fluid loss. This observation raises the possibility that pharmacologic agents which inhibit adenyl cyclase may prove effective in the treatment of cholera.

Because the formol inactivated toxoid proved to be unstable at room temperature and above, efforts have been concentrated on developing an improved and completely effective detoxification process which preserves maximum immunogenicity and prevents dissociation of the toxin from the toxoid complex. Preliminary studies with a glutoraldehyde inactivated toxoid appear promising, and a sufficient quantity of this material has been prepared for extensive animal and human testing. If the toxoid passes safety, potency and efficacy testing, field studies will be considered in the near future.

Studies of patients with non-vibrio cholera-like diseases, responsible for greater worldwide morbidity than cholera itself, show that at least two additional enteric bacteria, E. coli and Shigella, produce an enterotoxin which has effects qualitatively similar to those caused by the cholera enterotoxin. The possibility of developing a toxoid which will provide immunity against these newly recognized enterotoxins is currently being investigated. These findings may well enhance the basic knowledge of cholera as well.

LEPROSY

This past year, the U.S. Leprosy Panel has seen advances in pharmacology, immunology, and cultivation. In pharmacology, a three-year chemoprophylactic study in the Pingelapese people of the Pacific Trust Territory has culminated in the complete suppression of new leprosy cases. Acedapsone (DADDS), a respository type derivative of the venerable antileprotic drug, dapsone (DDS), was administered intramuscularly to the entire population at eleven-week intervals. No new cases were seen after the first year. This treatment has been terminated, except in known leprosy cases, and surveillance is planned for at least ten years. If it proves to be effective,over the long term, DADDS will significantly facilitate the therapy of leprosy patients in endemic areas with limited medical and public health capacity.

A new combination column chromatographic - fluorometric procedure has been developed for determining plasma levels of DDS, DADDS, and MADDS (monoacetyl DDS) in the 1-2 nanogram/ml range. The capability for sensitive analyses is essential for the rational therapeutic use of DADDS.

The drug, rifampin, has been found to kill the leprosy bacillus, Mycobacterium leprae, more rapidly in mice than any previous drug tested. However, it is expensive, as well as efficacious. Therefore, a combination treatment of rifampin and DADDS is currently being studied to reduce the cost of therapy as well as to reduce the likelihood of developing drug resistance to a single chemotherapeutic agent.

Progress in the area of immunology has continued. In an attempt to overcome the immunologic deficit of human lepromatous patients, single injections of transfer factor prepared from peripheral lymphocytes of normal persons and leprosy patients having positive lepromin reactions were used. A transitory flaring of lesions was observed. This preliminary information will provide a baseline for further studies dealing with the mechanism of lepromatous patients' immunity.

The success of the thymectomized-irradiated mouse as an animal model for leprosy research has stimulated exploration for improved animal models. Promising results have been obtained with the thymectomized-irradiated mouse in which the bone marrow of one limb is protected by a lead shield. Enhanced growth of M. leprae and reduced mortality have been observed. Results with neonatally thymectomized rats also appear favorable, especially after the rats have received anti-thymocyte serum.

Some encouraging reports have come from the continued efforts to cultivate the leprosy bacilli. This year evidence was obtained by radioisotopic techniques that M. leprae may multiply in cultures of lepromatous patients' macrophages.

In another study, M. lepraemurium was found to be capable of utilizing exogenous substrates related to the tricarboxylic acid cycle. A contract that is now in operation to supply skin biopsy specimens with large numbers of M. leprae should expedite cultivation research.

MALNUTRITION

Among current findings and major future priorities are:

Clarification of the mechanisms involved in the demonstrated correlation between retarded physical growth and development and the impaired performance of some tests of learning and behavior.

Study of the relationship among metabolic disturbances, anemia, lowered resistance to infection and mental retardation.

Clarification of the mechanisms responsible for the demonstrated reduction in resistance to infectious disease among even mildly malnourished individuals.

Determination of the quantitative significance of factors such as infection, increased sweat losses, altered gastric physiological adaptation to low dietary intakes which have been shown to increase nutrient requirements of underprivileged populations.

Investigation designed to prevent adverse consequences of the "Green Revolution" on protein nutritional status by improving the protein value of the new higher yielding cereals and reversing the alarming trend to decreased legume production and consumption in areas where the new cereal varieties have had their greatest success.

Research which clarifies the mechanism for changes in rate of protein biosynthesis in the liver of protein deficient animals.

PARASITIC DISEASES

Research under the Parasitic Diseases Panel is confined to two parasitic infections of very great importance in Asian countries - schistosomiasis and filariasis.

Schistosomiasis

Important advances were made during the year in the understanding of the mechanism of the immune response in schistosomiasis. The relation of immediate and delayed type hypersensitivity and granuloma formation around eggs in tissue was actively studied in experimental models. In addition to soluble antigens, cytotoxic materials such as lysophosphatides are released from eggs and may serve to initiate the primary granulomatous response. Schistosomes have also been found to incorporate host substance onto their integument to mask their own antigenicity and thus escape host immune response.

Analysis of the specific molecular conformational characteristics of anti-schistosomal, nitroheterocyclic compounds has led to accurate prediction of new compounds as being schistosomicidal, and a highly promising new drug (a nitrovinylfuran) has been discovered on this basis. In other pharmacologic studies, a disconcerting finding has been the documentation of the occurrence of resistance to the antischistosomal drug, hycanthone.

A workshop on the intramolluscan development of larval schistosomes has resulted in the definition of work for three contracts to develop mullusc cell line cultivation so that subsequent *in vitro* studies of schistosome infections can be carried out.

Filariasis

Small animal model systems for *Brugia pahangi* and *B. malayi* infections have been improved and partial success has similarly been obtained for *Wuchereria bancrofti*. These animal model systems are fundamental to experimental work on human filariasis.

There has been a continuing effort to synthesize antifilarial drugs, and these compounds have been thoroughly tested in animal models. A new contract to supply isotopically labeled diethylcarbamazine has stimulated several biochemists and physiologists to undertake studies of the mechanism of action of this important antifilarial compound.

Also in the area of chemotherapy, the enzyme dihydrofolate reductase has been found in adult forms of both filariids and schistosomes. Properties of the parasites' enzyme, when compared to those of the analogous mammalian enzyme, were sufficiently different to indicate a potential target for appropriate chemotherapeutic agents.

A workshop on the development of filariae in mosquitoes included discussions of pathology and defense mechanisms of the host, the effect of mosquito pathology on filarial infection, mosquito cell culture, inheritance of susceptibility to filarial infection in mosquitoes and quantitative aspects of filarial infections.

TUBERCULOSIS

In the past year significant advances have been made in the field of tuberculosis research through the cooperative efforts of a number of investigators. The chemical analysis of morphological constituents of tubercle bacilli and the biological characteristics of these constituents have been studied.

The role of lysosomes from macrophages, and from lung avelolar macrophages in particular, was studied in relation to growth inhibition of tubercle bacilli.

Microparticulate gel chromatography as a method for separating and identifying mycobacterial lipids, including cord factor, has been developed.

The mechanisms involved in the control that small lymphocytes exert on macrophages in immunity and delayed hypersensitivity have been investigated and extensively described.

Progress has been made in evaluating existing animal models for the study of tuberculosis vaccines. A sub-human primate model involving aerosol challenge has been developed and standardized.

From the mycobacterial culture bank materials such as PPD, antisera, and lyophilized BCG cells have been made available to investigators in the U.S. and Japan.

VIRAL DISEASES

Research under the Viral Diseases Panel is limited to studies on rabies and on arboviruses with emphasis on dengue and the pathogenesis of hemorrhagic fever.

Rabies

Antisera against eight strains of rabies virus and ten other rhabdoviruses have been prepared in rabbits and cross-testing carried out by cross-neutralization tests in mice, by the fluorescent antibody technique and by plaque reduction. Independently, the finding recently reported that rabies exhibits one way cross reactions with Lagos bat and Ibadan viruses was established by serum neutralization and fluorescent antibody procedures.

Studies on pathogenesis are in progress. Studies in spotted and striped skunks on pathogenesis and on viral replication in extra neural tissues of street virus are continuing using fluorescent antibody, histopathological and electron microscopic procedures.

Purification of rabies virus of rabbit brain origin has been achieved by exclusion chromatography on glass beads of controlled pore size. Substances of low molecular weight, such as host brain fragments, are retarded in their passage and emerge in a discrete zone after the infectious rabies virus, which travelled as a single narrow band, has been eluted.

During the past year, three contracts were awarded to fund research on the development of a more effective post-exposure rabies vaccine. The various aspects covered by these contracts involve development of techniques for inactivation of rabies virus, isolation of antigenic fractions and evaluation of vaccine potency. A workshop held in March 1972 afforded an opportunity for these contractors to exchange ideas and information in the development of a post-exposure vaccine.

Arboviral Diseases with emphasis on dengue

The antigenic relationship between dengue and yellow fever viruses, and the moderate protective effect of the former to challenge by the latter, suggests that the relative resistance of certain populations to yellow fever is due to the presence of dengue virus in those populations.

Passage of dengue virus in different hosts and/or cultures may selectively alter viral characteristics rendering vaccine strains more or less suitable for protection. In the case of type 1 virus, virulence as measured in the different test systems becomes restricted with mouse passage though enhanced reaction may be observed in species that remain susceptible.

Samples of sera from patients suffering from dengue shock syndrome were collected in Thailand and are being subjected to immunochemical analysis in an attempt to delineate the mechanism of shock development.

In September 1971, a joint U.S.-Japan conference was held on the pathogenesis of arboviral diseases. Mild arboviral syndromes, dengue shock syndrome and encephalitis were discussed in light of the contributions of epidemiology, clinical and pathological observations and experimental models to an understanding of pathogenesis.

Research Conferences and Workshops:

In addition to Program elements involving research grants and contracts, the U.S.-Japan Cooperative Medical Science Program includes a provision for regular scientific conferences and workshops. These include joint meetings with Japanese counterpart scientists as well as special conferences for American investigators working in the scientific areas of special interest to a given Panel. These workshops and conferences serve to stimulate and plan new scientific approaches to the six disease categories within the Program, and they also facilitate the timely exchange of research results, methods and protocols. The Joint U.S. and Japanese Panels meet annually, alternating between locations in the United States and Japan. A notation of conferences and workshops sponsored during FY 1972 follows:

Meeting Title and Location	Sponsoring Panel
July 26-28, 1971 Sixth Joint Leprosy Research Conference NIH, Bethesda, Maryland	Leprosy
July 26-28, 1971 Joint Parasitic Diseases Conference NIH, Bethesda, Maryland	Parasitic Diseases
August 9-11, 1971 Joint Cholera Conference Woods Hole, Massachusetts	Cholera
September 22-24, 1971 Conference on the Pathogenesis of Arboviral Infections, Honolulu, Hawaii	Viral Diseases
October 7-8, 1971 Cholera Workshop Johns Hopkins University, Baltimore, Maryland	Cholera
October 11-12, 1971 Mycobacterial Antigen Workshop San Francisco, California	Tuberculosis
October 13-14, 1971 Joint Tuberculosis Conference San Francisco, California	Tuberculosis
October 19-21, 1971 Joint Malnutriton Conference on Nutrition, National Development and Planning MIT, Cambridge, Massachusetts	Malnutrition
February 29 - March 1, 1972 Seventh Annual Leprosy Research Conference Menlo Park, California	Leprosy

Meeting Title and Location	Sponsoring Panel
March 7, 1972 Workshop on Rabies NIH, Bethesda, Maryland	Viral Diseases
March 20-21, 1972 Workshop on Development of Filariae in Mosquitoes, Los Angeles, California	Parasitic Diseases
May 5, 1972 Workshop on Mycobacterial Antigen Fractionation NIH, Bethesda, Maryland	Tuberculosis
June 26-27, 1972 E. coli Enterotoxin Workshop NIH, Bethesda, Maryland	Cholera

II. The International Centers for Medical Research and Training (ICMRT) Program

Program Description:

In response to the expressed desire of the Congress (P.L. 86-610), a program for the International Centers for Medical Research and Training was established in 1960 by the National Institutes of Health "to advance the status of the health sciences in the United States and thereby the health of the American people" by expanding collaborative research and research training between U.S. universities and selected foreign institutions and investigators.

The four grants funded under this Program are awarded for five years with continued support contingent upon program review at the end of each year of operation. One of the most important features of this program is that of long-term support, as contrasted with the transiency which has characterized a number of other international projects. This is a necessary ingredient for the full development of university careers in science which will effectively utilize the international medical experience of individual scientists. A major advantage of this program is that it provides opportunities for established and highly competent investigators to exploit valuable research opportunities abroad without losing their university affiliations and faculty appointments. The long-term support of the ICMRT's does not represent a form of foreign aid. Rather the primary objective is a collaborative effort in biomedical research and research training of specific relevance to the health of the American people and to the U.S. scientific community. The four current ICMRT grants were renewed for an additional five years beginning in FY 1970, and at the same time the NIH phased out ICMRT grant support of the LSU Center in San Jose, Costa Rica.

Purpose and Scope:

The ICMRT program has as its principal objective the provision of high quality research training and career motivation of Americans in the broad fields of

geographic medicine, and in response to the special opportunities existing within each ICMRT organization. The achievement of the research training goal for each ICMRT unit depends in large part upon the actual conduct of research by established investigators who can serve as mentors for those less experienced scientists participating primarily in a learning kind of international research experience. The length of the training period is a discretionary concern of the ICMRT program director, and is obviously determined by both the interests of the prospective scientist-trainee and the opportunities available within the given ICMRT unit. Consistently the emphasis is upon the quality rather than the quantity or duration of the research training experiences.

The aggregate ICMRT units are serving increasingly as a national resource for utilization by both trainee and trained scientists to create pool of investigators with a sustained career interest in international or geographic biomedical research. In this regard, the ICMRT program directors are encouraged to establish a selective, interdisciplinary scientific program. Their activities encourage and accommodate a number of parent university departments other than the department which primarily sponsors the ICMRT operation. Particular advantage is taken of special strengths within the overall parent university, including opportunities in health-related fields outside of the classical confines of tropical medicine.

During the FY 1971 the NIH initiated a new program element within the overall ICMRT framework. Designated as the ICMRT Research Associate Program (IRAP), this centrally directed activity is specially designed to recruit both experienced investigators and prospective trainees on a national basis and specifically from universities that do not have an ICMRT. The administrative structure of an ICMRT remains relatively flexible and is designed, constructed, and modified to complement in an optimum manner and build upon the sponsor's resources and the overseas opportunities and environment.

As far as is possible, the ICMRT core grant is used for training and research support at the off-shore ICMRT site rather than at the parent, domestic university. In this regard it is accepted that some training preliminary to an overseas assignment is frequently necessary or desirable, and as many items of equipment and supplies as feasible are purchased in the United States.

Program:

The four institutions participating in the ICMRT program at present, and their respective areas of research and training interests are as follows:

Johns Hopkins University (Baltimore, Maryland)

Program Director: Dr. Frederik B. Bang

Johns Hopkins University with the participation of the School of Hygiene and Public Health and the Department of Medicine is collaborating with the Calcutta School of Tropical Medicine, the All-India Institute of Hygiene and Public Health, and the Postgraduate Institute for Medical Education and Research in

Calcutta, India. Program emphasis is directed toward the epidemiology and management of human diseases of large populations and the ecology and behavior of animals which live in comparatively close association with man.

Studies are in progress on the year-round distribution of respiratory and enteroviruses in normal children in rural communities. Other research includes the factors influencing distribution of hookworm, the development of antigens for skin tests in leprosy, and the ecology of the economically important bandicoot rat (B. bengalensis).

Intensive biochemical research in cholera is also in progress. This work includes antibiotic and oral therapy, cholera in children, comparison of classical and El Tor cholera, and a number of very significant experimental pathophysiological studies.

University of California (San Francisco, California)

Program Director: Dr. J. Ralph Audy

The University of California with the participation of the Hooper Foundation, the School of Public Health, and the Medical School, is collaborating with the Institute for Medical Research, Kuala Lumpur, Malaysia, and the University of Malaya. This International Center continues to place strong emphasis on infectious diseases transmitted from animals to man. Because of the long standing research interest and experience in studying the fundamental problems of human ecology at the University of California, a collaborative program has been developed embracing medical zoology, microbiology and parasitology. The scope of this collaborative endeavor has now been expanded to include related socio-cultural research and medical genetics.

Long-term research projects in Malaysia and California are based on three general laboratory programs: the Arbovirus Research Unit, the Parasitology Laboratory, and the Medical Anthropology and Rural Health Research project.

The arbovirus research is focused on the epidemiology and ecology of dengue virus and the relationship of this virus to mosquito-borne Hemorrhagic Fever. The parasitological research is based on studies of trematode ecology and interactions of various parasites in the snail host and the ecology of Malayan pentastomes or tongue worms. The snail trematode antagonism studies are directed toward the biological control of schistosomiasis, and this ICMRT represents a leading world resource for this important field research. Investigators from this ICMRT have also studied the histopathology of host reaction to acarine parasites, the relationship of monkeys to human malarial infection, and jungle cycles of scrub typhus, Q fever, and tick typhus.

Abundance, distribution, life cycles and ectoparasites of rodents closely associated with man have been studied. Work on the possible role of the Indian fruit bat as a reservoir of human viral infections has been completed. Other field studies include the investigation of bacteria and viruses pathogenic for man in monkey populations. Socio-cultural studies concerned with Malayan attitudes toward health and nutrition and the influence of

native and western practitioners of medicine upon these attitudes have been undertaken by a number of variously trained, medically oriented social scientists.

University of Maryland (Baltimore, Maryland)

Program Director: Dr. David F. Clyde

The University of Maryland with the participation of its School of Medicine, and collaborating with the Institute of Hygiene, Lahore has undertaken a varied research program in West Pakistan. Comprehensive studies of arthropod-borne diseases have been undertaken. During the current year a field unit in Salvador (Bahia), Brazil was established at the Federal University of Bahia to study insect vector pathology as a tool for biologic control of malaria. Additional studies are planned in the area of schistosomiasis. Because long-term stability has become increasingly difficult to insure in Pakistan, it is likely that the University of Maryland will relocate their ICMRT from Pakistan to Brazil within the next 12-24 months.

More recent research in Pakistan includes studies of mosquito-borne viruses in man and a comprehensive epidemiologic survey of smallpox. In addition, a detailed investigation of several different clinical entities, e.g. bleeding disorders in humans, is now being completed. Research on immunity in tuberculosis and on the relationship between cirrhosis, Hepatitis Associated Antigen, and viral hepatitis is also near completion.

Mosquito genetic investigations of a comparatively unstudied vector of Japanese Encephalitis Virus have been of unusually high scientific merit and promise. Knowledge about the genetics of this mosquito, Culex tritaeniorhnychus now approaches the abundance and high quality of information previously known about two other mosquito vectors of human disease. This new information has been sought to establish a scientific basis for the ultimate biologic control of human Japanese encephalitis. A new "compound chromosome" technique has been developed during the past year and is under consideration for early field application.

Tulane University (New Orleans, Louisiana)

Program Director: Dr. Paul C. Beaver

Tulane University, with the participation of the Departments of Tropical Medicine and Public Health, other departments of the Medical School, and the Department of Anthropology and Sociology, is collaborating with the Universidad del Valle, Cali, Colombia. The research work is in two general areas: causes, prevalence and distribution of human diseases with human relevance, and physical environments which determine ill or well-being.

Parasitic diseases research has continued to focus principally around the following diseases: trypanosomiasis, malaria, filariasis, and amebiasis. The local epidemiology of Venzuelan Equine Encephalitis has been studied and documented following the recent epidemic. The Virus Group also has a continuing interest in rabies. A comprehensive clinical evaluation of bacterial

meningitis treatment regimens is in process. Finally, the multidisciplinary study of frustrated suicides has recently been continued and refined. New projects are concerned with maternal and child health, family planning, and include health services research.

GEOGRAPHIC MEDICINE BRANCH

2. CONTRACT NARRATIVES

U.S.-Japan Program

JEFFERSON MEDICAL COLLEGE (NIH 70-2188) .... Cholera

Title: Vibrio Reference Laboratory

Project Director: Dr. Harry L. Smith, Jr.

Project Officer: Dr. Earl S. Beck

Objectives: The objectives of this contract are: 1) to maintain a reference collection of Vibrio cholerae and other vibrios and to supply these strains upon request; 2) to receive and identify serologically new vibrio isolates; and 3) to prepare and distribute to qualified investigators cholera typing antisera.

Major Findings: The contractor meticulously maintains a reference library of some 5000 cultures of Vibrio cholerae and other vibrio-like strains. During the past year 100 cultures were received for typing and characterization, 632 vials of lyophilized cholera diagnostic sera were dispensed, and 15 cultures from the reference library were shipped out. The serum paper disc method described in last year's report is being evaluated by 20 investigators around the world, including WHO. Investigative work using sheep to produce diagnostic sera appears very promising, although the titer is somewhat lower than that observed in the rabbit, the increased volume obtained from the larger animal compensates for this reduction. Work is in progress to lyophilize all cultures in the collection.

Significance to NIAID Program and Biomedical Research: It is important in cholera research to have a laboratory which possesses the competence and the expertise to identify accurately and to differentiate V. cholerae from other enteric organisms. The need for reference cultures and for typing sera is important and essential to an active research program. Many investigators involved in the seventh cholera pandemic have used and depend upon the services and reagents of this laboratory.

Proposed Course of Project: The contract will remain in force as long as there is a demonstrated need for such a service.

Date Contract Initiated: June 1970

Current FY Funding Level: None

THE JOHNS HOPKINS UNIVERSITY (NIH 70-2051) .... Cholera

Title: Assays of Cholera Toxins and Toxoids in (A) Dogs and Rabbits and (B) Other Assay Systems

Project Director: Dr. Nathaniel F. Pierce

Project Officer: Dr. Robert S. Northrup (DBS)

Objectives: The purpose of this contract is to provide a service laboratory for the broad-based cholera research program. The primary objectives include the maintenance of laboratory and experimental animal facilities for the evaluation of purified cholera toxoids as immunizing agents against cholera, the development of improved assay techniques for the measurement of cholera toxoid and antitoxin activity, and the evaluation of immunizing techniques designed to achieve optimal antitoxin-mediated protection against cholera.

Major Findings: Dogs immunized with two injections of active cholera enterotoxin plus Freund's adjuvant showed significant protection for ten months against challenge with V. cholerae, Ogawa 395. This protection was, however, no longer evident at eighteen months. A clear-cut correlation between serum antitoxin titer and protection was demonstrated; titers greater than 100 Craig units afforded complete protection against lethal diarrhea. Immunization with three injections of Wyeth formalized toxoid produced lower antitoxin titers and provided protection of lesser magnitude and shorter duration than that provided by active enterotoxin plus Freund's adjuvant.

Because the formalin inactivated toxin proved to be unstable at room temperature and above, it was necessary to choose another detoxifying agent. Glutaraldehyde inactivated toxin preparations appear promising, and plans are to test the new toxoid in the various test systems.

Studies with Finkelstein's choleragenoid indicates that the moiety lacks the systemic toxicity associated with the active enterotoxin. Further studies suggest that choleragenoid competes with cholera enterotoxin for a common cellular binding site, while the formalinized cholera toxoid fails to show competitive inhibition of cholera enterotoxin.

Passive immunization studies have clearly demonstrated that antitoxin of serum origin is protective against intraluminal challenge with cholera enterotoxin.

Significance to NIAID Program and Biomedical Research: This facility will serve as a central testing laboratory for other investigators involved in cholera research. The service will provide uniform and rapid testing of submitted samples, thereby facilitating and expediting research toward the ultimate goal of developing an improved cholera toxoid.

Proposed Course of Project: The contractor serves a key function in a collaborative-type program designed to develop an improved cholera toxoid. It is anticipated that the contract will remain active until Program achieves or changes its guidelines.

Date Contract Initiated: December 1969

Current FY Funding Level: $72,000

THE RESEARCH FOUNDATION OF STATE UNIVERSITY OF NEW YORK (NIH 70-2091) ....
Cholera

Title: Establishment of a Laboratory for the Evaluation Measurement of Cholera Toxins, Antitoxins and Toxoids

Project Director: Dr. John Craig

Project Officer: Dr. Robert Northrup (DBS)

Objectives: Establishment of a laboratory to perform PF (permeability factor) titrations of cholera toxins, antitoxins and antitoxin combining power of cholera toxoids, toxins and sera. Also, to assay the immunogenicity of cholera toxoids by the mouse protection test, and the residual toxicity by mouse inoculation.

Major Findings: During the past year, the contractor continued to test cholera toxins, toxoids and sera for other investigators involved in a collaborative effort to develop an improved cholera vaccine. The instability of the formol-inactivated toxoid and the production of pilot lots of a new glutaraldehyde-inactivated toxoid have generated large numbers of samples to be tested in the various assay systems of the contract. A method for titrating cholera toxin in the suckling mouse has been developed and appears promising.

Significance to NIAID Program and Biomedical Research: The contractor will fulfill program need for comparative testing of materials under standard conditions for other research laboratories involved in cholera work.

Proposed Course of Project: It is anticipated that the contract will remain active until Program achieves or changes its guidelines.

Date Contract Initiated: April 1970

Current FY Funding Level: None

WYETH LABORATORIES, INC. (NIH 70-2102) .... Cholera

Title: Purified choleragenic toxins and vaccine (toxoid) production

Project Director: Dr. Benjamin Rubin
Dr. Alan Bernstein

Project Officer: Dr. Robert Northrup (DBS)

Objectives: The contractor will seek to convert purified toxin of standardized quality to stable toxoid with optimal immunogenicity. The pilot toxin lots will be further studied under a variety of detoxification procedures, singly or in combination, to achieve an irreversible product. Residual somatic antigen, if any, will be quantified by various tests. Practical adjuvanting methods will be sought in preparing test lots for clinical evaluation.

Major Findings: The toxin refinement process has been standardized to provide a purified product in good yield with as little as one-tenth or less of the residual somatic antigen content of comparison lot 00101, and the method can be reproduced on a manufacturing scale. Also, vibriocidal antibody responses in experimental animals were reduced by this process.

Preliminary studies with glutaraldehyde detoxification suggest that the problem of reversion to free toxin, characteristic of the formalinized product, has been solved. The glutaraldehyde toxoid has been successfully lyophilized and adjuvanted, and a sufficient quantity has been prepared for extensive animal and human testing. If the toxoid passes preliminary safety, potency and efficacy testing, field studies will be considered.

Significance to NIAID Program and Biomedical Research: The Program represents a collaborative effort of the Cholera Advisory Committee and the U.S.-Japan Program to develop an improved cholera vaccine using detoxified toxin. Also, testing of the toxoid in the field will help establish the present concept that known toxins are responsible for lethal diarrhea and that induced antitoxi can protect.

Proposed Course of Project: Review and evaluation for continued support prior to the anniversary date of the contract.

Date Contract Initiated: March 1970

Current FY Funding Level: $105,760

GULF SOUTH RESEARCH INSTITUTE (PH-43-68-1272) . . . Leprosy

Title: Analytical Methods for DDS, DADDS and Their Metabolites

Project Director: Dr. H. P. Burchfield

Project Officer: Dr. Paul D. Lambert

Objectives: A study was conducted by the contractor to develop an analytical method for the determination of nanogram levels of DDS and MADDS and their metabolites in plasma, urine, and tissue from human clinical subjects. The contractor used gas liquid chromatographic (GLC) analysis with electron capture detection.

Major Findings: The contractor has developed a method for converting DDS and MADDS to halogen derivatives that can be separated via gas-liquid chromatography and detected by electron capture. The method is sensitive enough for determining nanogram quantities of DDS and MADDS in plasma and urine. In an evaluation study of comparing the GLC method with a chromatographic-fluorometric method, with known samples in a double blind assay, the GLC method was found to be inadequate. DADDS was erroneously found to be present where no DADDS had been included in the sample. Since a chromatographic-fluorometric method was found superior to the GLC method, this contract effort was terminated.

Significance to NIAID Program and Biomedical Research: The ultimate objectives of this program were to develop methods for determining the metabolic pathways of DDS when administered at very low doses and to establish whether these were the same or different than those followed when DADDS and DDS were administered at high doses.

Proposed Course of Project: This project was terminated July 18, 1971

Date Contract Initiated: June 19, 1968

Current FY Funding Level: None

HARVARD COLLEGE (NIH 70-2285) . . . Leprosy

Title: Studies of Immunosuppression in Adult-thymectomized Mice Treated with Antilymphocyte Serum

Project Director: Dr. Anthony Monaco

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor proposes to study and define the course, duration and degree of immunosuppression in adult-thymectomized mice treated with antilymphocyte serum (ALS). A schedule for pulsed antilymphocyte serum therapy to maintain prolonged immunosuppression will be determined.

Major Findings: A major effort is continuing to define conditions for the production of a highly potent but non-toxic anti-lymphocyte serum which could be administered at intervals over a long period of time to adult thymectomized mice in order to achieve prolonged, perhaps lifelong immune suppression.

To follow this development of the immunosuppressed state in the animal, three techniques were employed: 1) skin allograft survival, 2) challenge with sheep red blood cells (SAR), and 3) graft-versus-host reaction. Skin allograft survival was found to be a function of graft placement relative to ALS treatment. Immune suppression as determined by the primary and secondary challenge with SRC paralleled to some degree that defined by skin allograft survival. To maintain suppression of the SRC hemagglutinin response, it was necessary to use a regimen of periodic ALS administration at dilutions which failed to maintain suppression of the allograft response. This finding indicates that to determine optimal regimen for ALS treatment, suppression of a very strong antigenic challenge for a very long period must be utilized as a criterion of effectiveness. Currently, a high dose antithymocyte serum (ATS) is available for use by leprosy investigators.

Significance to NIAID Program and Biomedical Research: Mycobacterium leprae cannot be grown in vitro, and its growth in vivo is so slow that experiments must be continued many months before an answer can be obtained. An animal model in which the organism would grow rapidly and produce disease similar to that in humans would do much to speed progress in leprosy research.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: June 1970

Current FY Funding Level: $33,477

UNIVERSITY OF HAWAII (NIH-72-2075) . . . Leprosy

Title: Leprosy Surveillance of Pingelapese Population

Project Director: Dr. Robert Worth

Project Officer: Dr. Paul D. Lambert

Objectives: To conduct scheduled examinations of the Pingelapese population in the Ponape District of the Eastern Caroline Islands to determine whether three years of mass chemoprophylaxis has broken the leprosy transmission cycle. Annual examinations are planned for 1972, 1973, and 1974.

Major Findings: The examinations of the Pingelapese people for 1972 has been completed, and a report of the findings is now in preparation.

Significance to NIAID Program and Biomedical Research: The results of continued surveillance of this unique population, treated prophylactically for leprosy, could have a substantial impact on the treatment and possible eradication of endemic leprosy throughout the world.

Proposed Course of Project: The contract will be reviewed for continued support at the end of the contract year.

Date Contract Initiated: January 1972

Current FY Funding Level: $7,500

LEONARD WOOD MEMORIAL (NIH-69-2003) . . . Leprosy

Title: Chemotherapy Trials in Leprosy

Project Director: Dr. Chapman H. Binford

Project Officer: Dr. Paul D. Lambert

Objectives: The Philippine Division of the Leonard Wood Memorial, Cebu City on the grounds of the Eversley Childs Sanitorium, has provided a leprosy research laboratory where clinical trials with drugs of interest can be performed. The Philippine government supports clinical care and management of leprosy patients, whereas studies associated with chemotherapy trials is being supported through research contracts. It is planned that strong bacteriological and pathological backup will continue to be provided by this research facility. Evaluation of several different treatment regimens is presently being provided utilizing the mouse footpad technique, and it is hoped that all animal studies will eventually be conducted at this facility. Because the facility is in a leprosy endemic area, untreated patients are admitted at a rate of approximately 4 per month and are available for chemotherapy trials now being conducted or to be considered at a later date. In addition, through the Philippine Health Department in Manila, skin biopsy material is being provided to U. S. investigators.

Major Findings: The B663 trials have progressed well, and it would appear that the daily and three times weekly regimens were more effective than the intermittent ones. All the data will be available within the next seven or eight months. Additional studies with DDS, DADDS, and rifampin, alone or in combination, are underway or planned. The DADDS - rifampin protocol is designed for 90 patients. Therefore, recruitment at the rate of 4 to 5 patients per month will require close to two years or perhaps longer. In addition, long-term follow-up studies on patients completing a clinical trial are suggested.

Significance to NIAID Program and Biomedical Research: This facility, in an endemic leprosy area, provides an important source of patients available to undergo clinical trials with anti-leprotic drugs.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.

Date Contract Initiated: March 18, 1969

Current FY Funding Level: $133,900

NATIONAL COMMUNICABLE DISEASE CENTER (67A-4) . . . Leprosy

Title: Studies on the Pharmacology of Leprosy and the Use of Immunosuppressed Mice in Leprosy Research

Project Director: Dr. Charles C. Shepard

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor will continue to study the activity of DDS and other anti-leprotic drugs using a pharmacokinetic method and employing the mouse footpad technique. Tests for the activity of drugs at different times during the growth period, with known drug dosages, will be used. In this manner drug effectiveness (bactericidal effect) will be determined. The contractor will also continue studies on the use of immunologically suppressed mice. Thymectomized irradiated and ALS treated mice will be investigated.

Major Findings: The contractor has found other drugs besides DDS and B663 to be bactericidal in the mouse footpad test system: Sulfadimethoxine, ethionamide, rifampin, streptovaricin and cephaloridine. Preliminary results indicate that rifampin is also effective in treating leprosy in man. However, the high cost of this drug may preclude its extensive use. Most antimalarial drugs studied were not active as antileprotic agents. Combination drug tests are now in progress utilizing the mouse footpad system, and these data will be particularly useful in studying the apparent emergence of bacterial resistance to DDS.

Significance to NIAID Program and Biomedical Research: By using kinetic methods of analysis, correlated with the mouse footpad technique, the effectiveness of drugs can be investigated in laboratory models before initiating clinical studies.

Proposed Course of Project: This project will be continued to investigate other drugs which appear to be effective against the leprosy bacillus.

Date Contract Initiated: May 15, 1967

Current FY Funding Level: $77,000

Title: Leprosy Biopsy Collection

Project Director: Dr. Roger A. Feldman

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor is to arrange for the collection and distribution of human skin biopsy specimens from leprosy endemic areas. These and other clinical materials will be made available to recommended research laboratories for further studies of interest to the Leprosy research program.

Major Findings: There is a reluctance on the part of clinicians, in leprosy endemic areas, to withhold patient treatment, prior to taking biopsies for shipment. Sources have been requested to insure that each shipment contain a minimum of 3 specimens from different individuals. Therefore, shipments are dependent upon having at least 5 to 10 leprosy patients seeking treatment weekly, with the expectation that a third of these will be untreated cases. Treatment could then be started with a minimum of delay. Most of the specimens received have been from areas with high incidence rate of untreated patients.

Biopsy specimens have been received from Ethiopia, Thailand, The Congo, Ghana and Korea. Thirteen shipments have been received containing approximately 36 biopsy specimens and these have been sent to six different investigators being supported under grant or contract by the program.

Significance to NIAID Program and Biomedical Research: Through the availability of clinical materials by this mechanism, it is hoped that clinical specimens can be obtained from patients which have not undergone any clinical treatment. Such untreated biopsy specimens are necessary for appropriate baseline studies in leprosy.

Proposed Course of Project: It is planned to continue the service now that sources have been developed.

Date Contract Initiated: January 1968

Current FY Funding Level: $4,000

OAKRIDGE NATIONAL LABORATORY . . . Leprosy

Title: To supply Thymectomized-irradiated Mice Treated with Bone Marrow to Investigators

Project Director: Dr. Charles C. Congdon

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor provides immunosuppressed adult thymectomized mice to leprosy researchers to investigate the usefulness of this animal model for the growth of M. leprae. In addition, the contractor studies the immune status of the irradiated animal.

Major Findings: The contractor has demonstrated marked defects in the immune response of thymectomized-irradiated mice chimeras. Anatomical studies of lymphatic tissues revealed a gross reduction in size of peripheral lymph nodes at one or two months after thymectomy. The germinal centers were almost completely absent in experimental mice at 1 month but were present in numbers equal to controls at 2 months. Lymphocytes were greatly reduced in the so-called thymus dependent areas of the spleen and lymph nodes at 1 and 2 months following treatment. However, some restoration of lymphocytes in the thymus-dependent region of the spleen was noted in the 2 months group. No residual thymus tissue was found in the thymectomized mice at autopsy. Tail skin allografts were rejected more slowly. The mean rejection time was 20 days compared to 10 days in normal control animals. Humoral antibody to rat red blood cells was not produced in significant amounts even after tertiary challenge.

Significance to NIAID Program and Biomedical Research: Mycobacterium leprae cannot be grown in vitro, and its growth in vivo in the mouse footpad model is so slow that many months are needed before evaluation of the assay method can be made. An animal model in which the organism would multiply more rapidly in significant quantities to provide the researcher with live bacilli would do much to speed progress in leprosy research.

Proposed Course of Project: The project will be funded as long as there is a need for the services provided.

Date Contract Initiated: July 1970

Current FY Funding Level: $47,500

STANFORD RESEARCH INSTITUTE (NIH-70-2283) . . . Leprosy

Title: Metabolism of Dapsone in Leprosy Patients

Project Director: Dr. John H. Peters

Project Officer: Dr. Paul D. Lambert

Objectives: In recent years leprosy researchers have determined that the principal metabolite of DDS in plasma, MADDS, is enzymatically acetylated by the same system which acetylates INH and sulfonamides. The speed at which a patient acetylates, slow acetylator vs. fast acetylator, in terms of therapeutic response of patients to DDS therapy, is not known at this time. To obtain additional information in this physiological response, leprosy patients in the Philippines, Japan, India and other countries will be classified as to acetylator phenotype using sufamethazine and DDS. Also, the effect of continued DDS therapy on metabolism of patients at the USPHS hospital in San Francisco will be investigated. Procedures will be initiated to increase the sensitivity of the fluorometric method to the one nanogram level for tissue specimens.

Major Findings: The contractor has determined that there appears to be a difference in acetylation rates in the Philippine population, and preliminary data indicate rate variation in a Congo population control. Preliminary data indicate that all DDS resistant patients are fast acetylators. This study has been performed on 5 patients and is continuing.

The contractor has established new contacts for phenotyping patients in Japan, India and an American Samoan population. Collaborative efforts will continue utilizing other populations.

In addition to the above studies, the contractor has developed a chromatographic fluorometric method for determining nanogram levels of DDS, MADDS and DADDS in plasma and urine samples. Efforts will be continued to develop this method for tissue specimen and analysis.

Significance to NIAID Program and Biomedical Research: Since DDS is the drug of choice for treating leprosy, it is important to learn as much as possible about the metabolism of this compound within the body. The study is providing useful data for future drug therapy.

Proposed Course of Project: The contract will be reviewed for continued support at the end of the contract year.

Date Contract Initiated: June 1970

Current FY Funding Level: None

STANFORD RESEARCH INSTITUTE (NIH-70-2286) . . . Leprosy

Title: Synthesis and Biological Evaluation of DDS Analogs and Sulfonamides

Project Director: Dr. Joseph I. DeGraw

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor plans to synthesize various simple analogs of dapsone, pteridine conjugates of certain sulfones, dapsone analogs modeled after sulfamethoxine and other compounds of close structural relationship to the sulfones to understand better the mechanism of action of DDS and its derivatives. The synthesized compounds will be evaluated *in vivo* and *in vitro*.

Major Findings: Good evidence has been accumulated indicating that sulfonamide and sulfone compounds do act via a related mechanism involving the incorporation of p-aminobenzoic acid (PABA) into dihydrofolate. The addition of either of the latter two compounds reversed the growth inhibitory action of both DDS and sulfamethoxolate.

In addition, the dihydrofolate reductase of the *Mycobacterium sp.*607 has been partially purified. This enzyme represents the second site of sequential blockade of the *de novo* mycobacterial folate pathway, and its inhibition by a variety of 2, 4-diaminopyrimidines has been studied. The combination of enzymic and whole cell inhibition studies will provide information regarding both inhibitory process and cell penetration ability.

Significance to NIAID Program and Biomedical Research: Dapsone is used world-wide in the treatment of leprosy and is at this time the drug of choice. By synthesizing and studying dapsone analogs and derivatives, an attempt will be made to relate chemical structure with drug activity. This information could ultimately be used to synthesize a more effective drug.

Proposed Course of Project: Review and evaluation at the end of the contract year for continued support.

Date Contract Initiated: June 1970

Current FY Funding Level: None

USPHS HOSPITAL, SAN FRANCISCO . . . . Leprosy

Title: Duplicate Mouse Inoculations of Skin Biopsy Specimens

Project Director: Dr. Louis Levy

Project Officer: Dr. Paul D. Lambert

Objectives: To continue to provide back-up testing and evaluation of the mouse footpad technique by performing duplicate mouse innoculations of skin biopsy specimens from leprosy patients in a chemotherapy trial. The specimens are shipped from the Leonard Wood Memorial Hospital in Cebu, Philippines to the PHS Hospital, San Francisco.

Major Findings: To date, there has been good correlation between the mouse footpad testing of the Cebu and San Francisco Laboratories. The monitoring of the San Francisco Laboratories is helping the Cebu Laboratory to gain experience in making the more difficult evaluations of tissues harboring fewer organisms as chemotherapy progresses.

Significance to NIAID Program and Biomedical Research: This project insures the reliability of results from a chemotherapy trial in leprosy patients.

Proposed Course of Project: The project will be funded as long as there is a need for the services provided.

Date Contract Initiated: July 1970

Current FY Funding Level: $19,000.

FOOD COMPOSITION TABLE FOR USE IN EAST ASIA . . . . Malnutrition

Intraagency Agreement - Nutrition Program, HSMHA

Contract - American Institute of Nutrition (NIH 70-2086)

Project Director: Dr. W. T. Wu Leung

Project Officer: Dr. B. T. Burton (NIAMD)

Objectives: To prepare a table of food composition for foods used in the East Asian countries of Burma, Thailand, Laos, Vietnam, Cambodia, Malaysia, Indonesia, Philippines, Taiwan, Korea and Japan. This includes indigenous food products as well as imported food-stuffs.

Major Findings: Food data are being collected through correspondence and by personal visits to the various countries. The information collected includes data on moisture, minerals, vitamins, amino acids, fatty acids, trace elements, local methods of preparation, processing and food preservation. Whenever feasible, local names used in various Far-Eastern countries with the corresponding English and scientific names are recorded and collected.

Significance to NIAID Program and Biomedical Research: The first Far-Eastern Food Table was issued in 1945 under the auspices of the Committee on International Food Value Programs of the National Research Council, U.S.A. Although it was revised and enlarged in 1952 as Agriculture Handbook No. 34, "Composition of Foods Used in Far Eastern Countries", it is not applicable to conditions in 1972. Comprehensive food tables for local foods in East Asia are essential for and basic to any attempt of improving the nutritional state of East Asian populations through a combination of nutrition education or nutrition improvement through introduction of innovations in the local food consumption.

Proposed Course of Project: It is expected that data collection will proceed at a reduced rate and will cease by late summer of 1972. Thereafter, tables will be assembled and coordinated. A final editing will be given to this material and the format during a joint meeting with the FAO counterparts in the late fall of 1972, and a printer-ready copy produced thereafter.

Date Initiated: HSMHA: - October 15, 1969
A.I.N. _ January 29, 1970

Current FY Funding Level: HSMHA - $10,600
A.I.N. - $39,118

HARVARD UNIVERSITY (NIH 68-1512) . . . . Malnutrition

Title: Study of Malnutrition and Mental Development in Bogota, Colombia

Project Director: Dr. Frederick J. Stare

Project Officer: Dr. Merrill S. Read (NICHD)

Objectives: To study effects of malnutrition on psychological development and mental functioning in children. To conduct a one-year study of sex-matched siblings in the slums of Bogota, Colombia. To analyze data obtained from nutritional, biomedical, psychological and social measurements. To evaluate the success of the nutrition intervention program and the methods of quantifying the data obtained with psychological tests which have been developed.

Major Findings: The effect of nutrition on mental development, learning and behavior has received increasing attention and research. Many studies have shown the coincidence of chronic malnutrition and mental retardation among under-privileged populations. Currently being examined is the question of whether malnutrition causes the mental retardation or whether other environmental factors related to poverty are of equal or greater importance. These studies involve selected pairs grouped into four categories of various degrees of nutrition. All members of the groups are receiving a supplemental diet aimed at achieving rapid recovery of the malnourished sibling and the prevention of malnutrition in the well nourished one. Clinical, psychological and biochemical tests are being carried out on these sibling pairs before and at various intervals during supplementation of the diet.

Significance to NIAID/NICHD Program and Biomedical Research: This contract represents a major attempt to develop a new study of nutrition and mental development with controls for genetics and the micro-environment of the family. It has been closely coordinated with other major studies on this subject.

Proposed Course of Project: The contract will terminate July 15, 1972. A no-cost extension for six months has been requested to permit completion of data analysis.

Date Contract Initiated: June 1968

Current FY Funding Level: $75,000

INSTITUTE OF NUTRITION OF CENTRAL AMERICAN AND PANAMA (INCAP) (NIH NICHD 72-2737)

. . . . Malnutrition

Title: Analysis of Data from an Ecological Study of Infection, Malnutrition and Growth of Children in a Guatemalan Indian Village

Project Director: Dr. Leonardo J. Mata

Project Officer: Dr. Merrill S. Read (NICHD)

Objectives: To analyze and interpret data from a longitudinal study of nutrition, infection and growth among pregnant and lactating women, young children and infants in a small village in Guatemala.

Overall, four study objectives have been identified:

1. Elucidation of the relationships between gestational events and growth and infection in the first year of life.

2. Elucidation of the post-partum relationships between infection, nutrition and growth.

3. Identification of selected viral isolates from fecal samples in storage as needed.

4. Preparation of a monograph which will describe and summarize the collected data as well as present analytic results and interpretations.

Major Findings: It is too early in the project to report findings.

Significance to NIAID/NICHD Program and Biomedical Research: The majority of the world's people live under poverty conditions where both malnutrition and frequent illness exist together. It has been suggested that malnutrition and infection have a synergistic deleterious effect on child growth and development. The data to begin answering this question are being analyzed under this contract.

Proposed Course of Project: Total of two years.

Date Contract Initiated: April 1, 1972

Current FY Funding Level: $73,000

INTERNATIONAL RICE RESEARCH INSTITUTE (PH-43-67-726) . . . . Malnutrition

Title: Improvement of the Protein Content of Rice

Project Director: Dr. B. Juliano

Project Officer: Dr. B. T. Burton (NIAMD)

Objectives: To determine the feasibility of incorporating high level protein strains into commercially produced rice in Asia. To determine the amino acid profile of the new rice protein. To determine protein efficiency ratios in rats and to carry out nitrogen balance studies in man.

Major Findings: The International Rice Research Institute rice collection (7500 varieties) has been analyzed for protein content and 100 contained at least 13% as compared to 7% for commercial rice. Selected high protein varieties were crossed with successful ideal commercial varieties and the high protein quality was incorporated permanently into several sequential generations. This new rice is now being tested for biologic value as compared with regular commercial rice. Early experiments indicate it sustains a positive nitrogen balance in young adults in contrast to regular rice which cannot.

Significance to NIAID Program and Biomedical Research: Ordinary commercial rice does not contain enough protein to support normal growth and development of infants and children maintained on a predominant rice diet, and does not protect the adult population from serious protein under-nutrition. Rice is the principal food of 60 percent of mankind. If the present protein level of rice (approximately 7-1/2 percent) could be elevated by several percentage points, an automatic and sweeping nutritional improvement would take place in all the populations for whom this one crop represents the nutritional mainstay. Such a development would concomitantly relieve the current burden of the United States which supplies hundreds of millions of dollars' worth of supplementary foods to underdeveloped rice-eating countries. The potential nutritional improvement among Asian populations and under-developed rice-eating countries throughout the world is an important aim of the U.S.-Japan Program and also of the applied nutrition program of the NIAMD which has scientific cognizance over the malnutrition portion of the U.S.-Japan Program.

Proposed Course of Project: Cross-breeding experiments will continue to select out high protein strains with improved field qualities. In the face of the encouraging progress thus far, further cross-breeding and growing of the resulting hybrids in large plots is contemplated in conjunction with additional protein efficiency ratio determinations in rats. Human nitrogen balance and digestibility studies of the protein of the resulting hybrid crops will be carried out.

Date Contract Initiated: May 10, 1967

Current FY Funding Level: $44,064

UNIVERSITY OF AKRON (NIH-70-2273) . . . Parasitic Diseases

Title: Development of a Toxic Bait for Destruction of Biomphalaria glabrata and Other Snail Vectors of Schistosomiasis

Project Director: Mr. Nathan Cardarelli

Project Officer: Dr. Kenneth Phifer

Objectives: To formulate and manufacture on a pilot scale a toxic bait for the control of the snail vectors of schistosomiasis. The toxic bait combines the slow release of a snail attractant with a molluscicide which kills the snail following ingestion. Those formulations which have been effective in laboratory screening will be evaluated in field test sites under a variety of environmental conditions.

Major Findings: Baits appear to be a feasible means of killing the snail, Biomphalaria glabrata in the field. Trials conducted in St. Lucia demonstrated that baits can destroy over 70% of the snail population in five days. One of the major advantages of this approach is its lack of toxicity for other organisms present in snail habitats.

In the initial field tests, baits hardened due to the chemical properties of the particular hard waters involved. By reformulation with flexibilizing agents this problem was ameliorated.

Significance to NIAID Program and Biomedical Research: Control of the snail vectors of schistosomiasis is highly relevant to the mission of the U.S.-Japan Program. There is great need for the development of molluscicides with residual activity since biological control of these vectors is still a goal of the future.

Proposed Course of Project: This project terminated March 31, 1972

Date Contract Initiated: June 26, 1970

Current FY Funding Level: None

UNIVERSITY OF GEORGIA (NIH-69-92) . . . Parasitic Diseases

Title: Filariasis Repository and Chemotherapy

Project Director: Dr. Paul Thompson

Project Officer: Dr. Kenneth Phifer

Objectives: To maintain colonies and life cycles of vectors and parasites for several species of filariae to be used in medical research; to supply qualified investigators with filariasis research materials in response to authorized requests; to evaluate selected compounds for antifilarial activity in experimental animals.

Major Findings: During the past year 67 requests were filled supplying filariae and vectors to investigators working on the immunology, chemotherapy, metabolism, fine structure and host-parasite relationships of filariae. Twenty-nine compounds were evaluated for antifilarial activity. Fourteen of these showed activity against either the microfilariae or adult worms of L. carinii.

Significance to NIAID Program and Biomedical Research: Basic research in filariasis has been retarded by the difficulty inherent in the maintenance of the life cycles of filarial parasites. The supply of filarial material has encouraged immunologists, biochemists, pharmacologists, etc., to undertake needed studies in this area. In addition, there has been a need for an intensified search for better chemotherapeutic agents. The contractor has been able to apply both his expertise and his laboratory facilities to this aspect of filariasis research.

Proposed Course of Project: This project will be funded as long as there is a need for the services provided.

Date Contract Initiated: January 22, 1969

Current FY Funding: $63,771

UNIVERSITY OF MICHIGAN (PH-43-67-736). . . Parasitic Diseases

Title: Supplying Animals Infected with Three Human Schistosomes

Project Director: Dr. Elmer Berry

Project Officer: Dr. Kenneth O. Phifer

Objectives: To maintain colonies and life-cycles to supply parasites, infected animals and intermediate hosts of the three human schistosome species and other schistosome materials to selected investigators.

Major Findings: Improved methods of culturing the snail hosts and infecting the definitive hosts, as well as increasing the numbers of geographical races to five each of S. mansoni, S. japonicum and S. hematobium allowed the contractor to supply qualified investigators with materials needed for research.

In the past year, 168 shipments were made to 32 investigators. Included in these shipments were 8600 infected rodents and 1600 infected snail vectors. In addition, the contractor's staff provided short-term training in routine procedures of snail maintenance to investigators from seven countries.

Significance to NIAID Program and Biomedical Research: Because of substantial technical difficulties in maintaining schistosome life cycles in the laboratory, the supply of this material enables a number of qualified investigators to carry out research in the field of schistosomiasis. If each investigator had to maintain complete life cycles of this parasite, very few laboratories would be engaged in schistosomiasis research. Since this disease is second in terms of world-wide morbidity, infecting over 200 million people, research stimulated by the availability of schistosome material is definitely of biomedical significance.

Proposed Course of Project: To continue to supply schistosome material to investigators now working in the field and to stimulate additional investigators to enter this area of parasitological research.

Date Contract Initiated: April 12, 1967

Current FY Funding Level: $75,243

STANFORD RESEARCH INSTITUTE (NIH-71-2122) . . . Parasitic Diseases

Title: Synthesis of Labeled Diethylcarbamazines

Project Director: Mr. Morris Leaffer

Project Officer: Dr. Kenneth O. Pfuifer

Objectives: To prepare three separate isotopically labeled diethylcarbamazines. These products were prepared in three gram quantities each and packaged in 100 mg. lots. The products are being distributed to competent investigators upon request.

Major Findings: Several investigators have been sent 100 mg. amounts of labeled diethylcarbamazine for use in their research on the mechanism of action of this antifilarial compound.

Significance to NIAID Program and Biomedical Research: There is a dearth of knowledge in the field of filariasis chemotherapy. Much work needs to be done on the metabolism of diethylcarbamazine, the current drug of choice, since little is known concerning its mechanism of action. This source of isotopically labeled drug will enable investigators to elucidate the metaboli fate of diethylcarbamazine and to make possible the synthesis of analogues leading to more effective antifilarial compounds.

Proposed Course of Project: The contract terminated in March 1972 but arrangements are being made for the storage and distribution of the remaining labeled material.

Date Contract Initiated: March 22, 1971

Current FY Funding: None

HAZELTON LABORATORIES (NIH-70-2242) . . . Tuberculosis

Title: Study of BCG Vaccination of Subhuman Primates

Project Director: Dr. Robert C. Good

Project Officer: Dr. Paul D. Lambert

Objectives: To compare the effectiveness of different routes of administration of BCG vaccine on protection of nonhuman primates against aerosol challenge with virulent tubercle bacilli.

Major Findings: Nonvaccinated rhesus monkeys developed progressive tuberculosis following an aerosol challenge with 22 viable units of virulent tubercle bacilli. Intracutaneous vaccination of rhesus monkeys with BCG did not prevent development of disease following challenge, but the progress of disease in these monkeys was slowed and more limited than that in nonvaccinated monkeys. Intravenous injection or inhalation of the vaccine provided significant protection against an aerosol challenge. The intravenous route appeared to give the best protection, but disease in monkeys vaccinated by inhalation of aerosol was rare and limited. However, the intravenous dosage of vaccine was four times greater than that administered by inhalation, and this difference may well account for the disease observed in the latter. Marked tuberculin hypersensitivity, which was evident in the control group after challenge, was not a significant indicator of disease in any of the vaccinated groups. Weak allergic reactions in the post-vaccination period did not become more intense in the postchallenge period.

Significance to NIAID Program and Biomedical Research: Because of varied reports in the scientific literature concerning efficacy of tuberculosis vaccines in animal systems, the Program wanted to develop a standardized test method suitable for evaluating vaccines in animals. From this first contract animal study, it would appear that this model shows promise for further exploration.

Proposed Course of Project: The contract terminated March 31, 1972

Date Contract Initiated: June 1970

Current FY Funding Level: None

HAZELTON LABORATORIES-(TRW) (NIH-71-2349) . . . Tuberculosis

Title: Preparation of Mycobacterial Products

Project Director: Dr. Robert Good

Project Officer: Dr. Paul D. Lambert

Objectives: To prepare the following mycobacterial products:

1. Antigen prepared from approximately 80 liters of culture filtrate
2. Dry bacilli
3. Cell extract prepared from organisms grown in 10 liters of media

Major Findings: After a successful scale-up from pilot runs, the contractor produced and is preparing the specified bacterial products for delivery.

Significance to NIAID Program and Biomedical Research: The reference system to be developed using the above products, has the potential of providing a means to identify species-specific antigens, to develop an antigenic taxonomy for the mycobacteria, to identify antigens important in immunity to tuberculosis, and to provide a functional basis of communication for investigators in mycobacteriology.

Proposed Course of Project: The contract will terminate June 1972

Date Contract Initiated: June 1971

Current FY Funding Level: $4,850

NATIONAL JEWISH HOSPITAL(NIH-70-2079) . . . Tuberculosis

Title: Serological Identification of "Atypical" Mycobacteria

Project Director: Dr. Werner B. Schaefer

Project Officer: Dr. Paul D. Lambert

Objectives: The contractor will continue to maintain reference cultures and prepare specific antisera to approximately 28 "atypical" mycobacterial serotypes and supply recommended investigators with these reagents upon approval of the Project Officer. He will also continue work on the serological identification of unclassified strains and improvement of pertinent serological techniques. In addition, he will serve as a reference laboratory for the serology of "atypical" mycobacteria, and to assist the Trudeau Institute in the characterization of reference strains which are to be maintained in the Trudeau Culture Collection.

Major Findings: Over 300 strains from various laboratories throughout the world have been studied and identified. For example, three strains of M. avium and a M. intracellulare, serotype Davis, were recovered and identified from patients with disseminated disease. The latter strain and M. serofulacium, serotype Lunning, were identified in hospital clinical specimens. Several new serotypes of M. intracellulare have been identified and are being added to the collection. Fifty-seven strains of the Runyon Group III, assembled by Dr. G. Meissner, have been serotyped. Presently, 59 strains assembled by Sr. Saito (Japan) are being investigated. An improved technique preabsorbing the homologous sera with bacilli of the heterologous type, has reduced the incidence of cross reactions.

Significance to NIAID Program and Biomedical Research: A high priority has been placed onthe maintenance and standardization of mycobacterial species of clinical significance by the U.S. Tuberculosis Panel. The research and reference laboratory of the National Jewish Hospital with "atypical" mycobacteria augments the culture bank and investigations of the Trudeau Institute with "typical" strains of mycobacteria. Reference laboratories provide numerous services and scientific information necessary for improved research.

Proposed Course of Project: This project will be continued as long as there is a need for the services required.

Date Contract Initiated: February 17, 1970

Current FY Funding Level: $34,284

TRUDEAU INSTITUTE, INC. (PH 43-68-640) . . . Tuberculosis

Title: Authentication, Maintenance, and Distribution of Mycobacterial Cultures

Project Director: Dr. George P. Kubica

Project Officer: Dr. Paul D. Lambert

Objectives: To characterize, distribute and maintain "prototype" mycobacterial strains.

Major Findings: Approximately 136 of 150 strains of mycobacteria have been authenticated both in vitro and in vivo by growth characteristics, animal pathogenicity, drug susceptibility, and other biological parameters. One hundred thirty cultures have been characterized and packaged in 7H-9 Middlebrook media for storage at -70°C and about 1200 cultures have been distributed to 72 requestors. A catalog of all available strains has been prepared.

Significance to NIAID Program and Biomedical Research: The contractor maintains a highly pedigreed collection of mycobacterial cultures which are available to competent investigators through the U.S.-Japan Program. Reference reagents, such as PPD, antigen and antiserum are reposited at and distributed by the contractor. The use of characterized cultures and standard reference reagents has already done much to develop research comparability from one laboratory to another in the United States and abroad.

Proposed Course of Project: The contract will be continued as long as Program determines there is an established need by competent investigators for highly characterized cultures and for other reference materials, such as PPD and mycobacterial antigen preparations.

Date Contract Initiated: February 12, 1968

Current FY Funding Level: $57,820

UNIVERSITY OF WISCONSIN (NIH-69-2175) . . . Tuberculosis

Title: Development of a Test System for Evaluating Tuberculosis Vaccines

Project Director: Dr. Donald W. Smith

Project Officer: Dr. Paul D. Lambert

Objectives: To develop a standardized test methodology by which tuberculosis vaccine potency can be determined before these vaccines are subjected to clinical trials.

Major Findings: The contractor has utilized a multivariate analysis which includes an orthogonal, individual degrees-of-freedom technique in an attempt to identify those variables of a test system that may influence the ranking of potencies of tuberculosis vaccines. Results at present infer that many factors influence the outcome of any single animal model; and that no satisfactory test system is currently available for evaluating the usefulness of a vaccine as it might be applied in humans.

Significance to NIAID Program and Biomedical Research: Because of variations in methodology, it has not been possible to compare vaccination test results from one laboratory to those of another laboratory. The contractor attempted without success, to devise a method which could be used to evaluate tuberculosis vaccines in guinea pigs or mice. Data generated by another contractor using a primate model for this evaluation appears more promising.

Proposed Course of Project: The contract terminated September 30, 1971

Date Contract Initiated: June 1969

Current FY Funding Level: None

STATE OF MICHIGAN DEPARTMENT OF PUBLIC HEALTH (NIH-71-2199) . . . Viral Diseases

Title: Work on an Improved Method for Inactivation of Rabies Virus Without Significant Loss of Immunogenicity

Project Director: Dr. John Mitchell

Project Officer: Dr. Kenneth O. Phifer

Objectives: To develop a safe and effective procedure for the inactivation of rabies virus. The inactivating agent, beta-propriolactone, will be compared with formalin in determining the kinetics of rabies virus inactivation and the effect of the agent on rabies virus antigen. To test for completeness of inactivation and residual levels of inactivating agent.

Major Findings: Degradation kinetics of beta-propriolactone have been defined and it has been shown that only four molar equivalents of sodium thiosulfate are required to effect complete neutralization of the inactivating agent. Highly sensitive procedures have been developed to determine levels of residual live rabies virus after inactivation. This technique is up to 1000 times more sensitive than direct mouse inoculation.

Significance to NIAID Program and Biomedical Research: Current rabies vaccines do not produce reliable protection and often evoke paralytic accidents. Thus, strong and lasting immunity in a short period of time with few inoculations. One aspect of the development of an improved vaccine involves the improvement of methods of inactivation of rabies virus without a loss of immunogenicity.

Proposed Course of Project: The aims of the project are expected to be reached at the end of this year at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 29, 1971

Current FY Funding Level: None

UNIVERSITY OF MICHIGAN SCHOOL OF PUBLIC HEALTH (NIH-71-2204) . . . Viral Diseases

Title: Isolation and Characterization of Protective Antigens of Rabies Virus

Project Director: Dr. Edward Eckert

Project Officer: Dr. Kenneth Phifer

Objectives: To isolate, concentrate and identify the essential protective antigens of rabies virus in order to obtain a highly refined and more effective post-exposure vaccine for use in man. This involves the isolation and characterization of protein and glycoprotein sub-units of rabies virus. Another objective of this study will be to evaluate the antigenic potency of sub-unit rabies vaccines and the role of the physical state of the antigens and adjuvants on immune response.

Major Findings: Various procedures for purification of rabies virus have been developed. It was found that polyethylene glycol precipitation was useful in isolating both intact virus and soluble antigen. Acrylamide gel electrophoresis is being tested for efficacy in isolation of purified protective antigen.

Significance to NIAID Program and Biomedical Research: One of the principal objectives of the U.S.-Japan Program is the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine. The standard rabies treatment involves a long series of inoculations of vaccines which do not provide reliable protection and which may evoke paralytic accidents. One of the most relevant areas of endeavour is the isolation and identification of the essential protective antigens of rabies virus with the ultimate objective of developing a sub-unit vaccine which produces strong and lasting immunity in a short period of time after a minimum number of inoculations.

Proposed Course of Project: The immediate aims of the project are expected to be reached in three years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 29, 1971

Current FY Funding Level: None

UNIVERSITY OF TEXAS MEDICAL SCHOOL AT SAN ANTONIO (NIH-69-2049) . . .
Viral Diseases

Title: Purification of Rabies Vaccine by Exclusion Chromatography on Glass of Controlled Pore Size

Project Director: Dr. C. W. Hiatt

Project Officer: Dr. Kenneth O. Phifer

Objectives: To devise a commercially feasible method for purifying rabies vaccines of brain origin by removing the specific impurities which cause allergic encephalitis. To develop and improve the technology or virus purification by steric exclusion chromatography on porous glass.

Major Findings: It has been demonstrated that exclusion chromatography on porous glass powder is a feasible method for isolating rabies virions from lower-molecular-weight constituents of infected brain tissue. The key to success of the method is the ability to block absorption of the virus by pre-conditioning the glass surfaces with polyethylene oxide. The premises underlying these studies is that it is desirable to isolate the rabies virion from the low molecular weight substances in mammalian brain tissue, including those myelin polypeptides known to have encephalitogenic properties.

Significance to NIAID Program and Biomedical Research: Present rabies vaccines contain undesirable constituents which are not part of the virus. This technique has proven effective in the purification of other virus and thus should prove equally fruitful with rabies virus. In addition, since the use of brain tissue vaccines is still being widely used in Asia, this project is quite relevant to the mission of the U.S.-Japan Program.

Proposed Course of Project: The contract terminated December 31, 1971

Date Contract Initiated: May 13, 1969

Current FY Funding Level: None

THE WISTAR INSTITUTE (NIH-71-2292) . . . Viral Diseases

Title: Improved Methods for Potency Testing of Standard and Refined Rabies Vaccines

Project Director: Dr. Tadeusz Wiktor

Project Officer: Dr. Kenneth O. Phifer

Objectives: To develop simple and economical procedures for the evaluation of the protective activity of rabies vaccines by means of an *in vivo* test in which treatment will follow the virulent virus challenge. To develop *in vitro* procedure for a rapid determination of the protective activity of such vaccines. To determine which of the presently available serological assays for rabies antibody best measures the state of resistance to rabies infection.

Major Findings: An experimental hamster model system was developed for evaluation of the potency of rabies vaccines in post-exposure treatment. It was found that interferon induction by inactivated rabies vaccines may play an important role in the mechanism of protection in the post-exposure treatment.

Significance to NIAID Program and Biomedical Research: The U.S.-Japan program considers relevant the encouragement of research on rabies with emphasis on studies leading to an improved post-exposure rabies vaccine since currently available vaccines represent one of the crudest and most unsatisfactory biological products in use in man. One aspect of the development of an improved post-exposure vaccine involves the improvement of methods of potency testing of standard and refined vaccines since existing procedures do not adequately characterize vaccines of tissue culture origin.

Proposed Course of Project: The immediate aims of the project are expected to be reached in from two to four years at which time the project will be reviewed regarding further development.

Date Contract Initiated: June 1, 1971 .

Current FY Funding Level: $30,287

CHOLERA BIBLIOGRAPHY

1. Banwell, J.G., Shepard, R., Thomas, J., Pierce, N.F., Mitra, R.C., Gorbach, S.L., Brigham, R.L., Fedson, U.S., and Mondal, A. Net and unidirectional transmucosal flux of sodium and water in acute human diarrhea disease. J. Lab. Clin. Med. In press.

2. Pierce, N.F., Graybill, J.R., Kaplan, M.M., and Bouwman, D.L. Systemic effects of parenteral cholera enterotoxin in dogs. J. Lab. Clin. Med. 79: 145-156, 1972.

3. Pierce, N.F., Kaniecki, E.A., and Northrup, R.S. Antitoxic protection against experimental cholera. J. Inf. Dis. In press.

4. Pierce, N.F. and Wallace, C.K. Stimulation of jejunal secretion by a crude E. coli enterotoxin. Gastroenterology. In press.

5. Sack, G.H., Jr., Pierce, N.F., Hennessey, K.N., Mitra, R.C., Sack, R.B., and Guka Mazumder, D.N. Gastric acidity in cholera and non-cholera diarrhea. Bulletin World Health Organization. In press.

6. Smith, H.L. and Mandle, R.J. A method for the preservation of diagnostic sera for field and laboratory work. Applied Microbiology 21: 783-785, 1971.

7. Van Heyningen, W.E., Carpenter, C.C.J., Pierce, N.F., and Greenough, W.B. Deactivation of cholera toxin by ganglioside. J. Inf. Dis. 124: 415-418: 1971.

8. Zieve, P.D., Pierce, N.F. and Greenough, W.B. Stimulation of glycogenolysis by purified cholera exotoxin. Johns Hopkins Med. Jour. 129: 299-303, 1971.

LEPROSY BIBLIOGRAPHY

1. Congdon, C.C., Payne, H., and Gengozian, N. Immune response in thymectomized chimeras. Sixth Joint Leprosy Research Conference, July 26-28, 1971 Bethesda, Maryland. Int. J. Leprosy. In press.

2. Congdon, C.C., Payne, H., Gengozian, N., and Usso,P. Immune status at twelve months in thymectomized radiation chimeras. Seventh Annual Leprosy Research Conference, February 29 - March 1, 1972, Menlo Park, California Int. J. Leprosy. In press.

3. Gelber, R., Peters, J.H., Gordon, G.R., Glazko, A.J. and Levy, L. The polymorphic acetylation of dapsone in man. Clin.Pharm. and Therap. 12: 225-238, 1971.

4. Gordon, G.R., Murray, J.F., Jr., Ghoul, D.C., Peters, J.H., Fieldsteel, A.H., and Levy, L. Rats in leprosy research: metabolic disposition of dapsone in normal and infected animals. Seventh Annual Leprosy Research Conference, Menlo Park, California, February 29 - March 2, 1972. Int. J. Leprosy. In press.

5. Gozzo, J.J., Wood, M.L., and Monaco, A.P. Use of minimal doses of lymphoid cells for production of heterotogous antilymphocyte serum. Transplantation Proc. 3: 779-783, 1971.

6. McRae, D. and Shepard, C.C. Relationship between the staining quality of Mycobacterium leprae and infectivity for mice. Inf. and Immunol. 3: 116-120, 1971.

7. Murray, J.F., Jr., Gordon, G.R., and Peters, J.H. A chromatographic-fluorometric procedure for the determination of nanogram quantities of anti-leprotic sulfones. J. Lab. Clin. Med. 78: 464, 1971.

8. Peters, J.H., Gordon, G.R., Karat, A.B.A., and Meyers, W.M. Metabolic disposition of dapsone in Indian and African subjects. Seventh Annual Leprosy Research Conference, Menlo Park, California, February 29 - March 2, 1972. Int. J. Leprosy. In press.

9. Peters, J.H., Gordon, R.G., Ghoul, D.C., Tolentino, J.G., Walsh, G.P., and Levy, L. Studies on the disposition of dapsone in Philippine subjects. Clin. Pharmacol. Ther. In press.

10. Russell, D.A., Shepard, C.C., McRae, D.H., Scott, G.C., and Vincin, D.R. Treatment with 4-4' diacetyldiaminodiphenylsulfone (DADDS) of leprosy patients in the Karamui, New Guinea. Am. J. Trop. Med. and Hyg. 20: 495-501, 1971.

11. Shepard, C.C. and McRae, D.H. Hereditary characteristics that vary among isolates of Mycobacterium leprae. Inf. and Immunol. 3: 121-126, 1971.

12. Shepard, C.C., Walker, L.L., Van Landingham, R.M. and Redus, M.A. Kinetic testing of drugs against Mycobacterium leprae in mice. Am. J. Trop. Med. and Hyg. 20: 616-620, 1971.

13. Sloan, N.R., Worth, R.M., Jano, B., Fasal, P., and Shepard, C.C. Repository acedapsone in leprosy chemoprophylaxis and treatment. Lancet 2: 525-526, 1971.

MALNUTRITION BIBLIOGRAPHY

1. Bressani, R., et al: Evaluation of the protein quality and milled rices differing in protein content. Agricultural and Food Chemistry, October, 1971.

PARASITIC DISEASES BIBLIOGRAPHY

1. Thompson, P.E. Perspectives and important needs in parasite chemotherapy. J. of Parasitology 57: 3-8, 1971.

TUBERCULOSIS BIBLIOGRAPHY

1. Janicki, B.W., Chaparas, S.D., Daniel, T.M., Kubica, G.P., Wright, G.L., Jr., and Yee, G.S. A reference system for antigens of mycobacterium tuberculosis. Am. Rev. of Resp. Dis. 104:4, 1971

2. Marks, J., Jenkins, P.A., and Schaefer, W.B. Thin layer chromatography of mycobacterial lipids as an aid to classification. Tubercle, Lond., 52: 219-225, 1971.

3. Ribi, E., Anacker, R.L., Barclay, W.R., Brehmer, W., Harris, S.C., Leif, W.R., and Simmons, J. Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the rhesus monkey. J. Inf. Dis. 123:5, 1971.

4. Smith, D.W., Harding, G.E., Wiegeshaus, E.H., Grover, A.A., and McMurray, D.N. Variables in tuberculosis vaccine test systems - a preliminary report. Int. Symposium on BCG Vaccine, 17: 259-268, 1971.

5. Smith, D., Wiegeshaus, E., Stark, R., and Harding, G. Models for Potency Assay of Tuberculosis Vaccines. Conference on Immunization in Tuberculosis In press.

6. Wiegeshaus, E., Harding, G., McMurray, D., Grover, A., and Smith, D.W. A cooperative evaluation of test systems for evaluation of test systems used to assay tuberculosis vaccines. Bulletin World Health Organization, 45:543-550, 1971.

VIRAL DISEASES BIBLIOGRAPHY

1. Aaslestad, H.G. and Urbano, C. Nucleotide composition of the ribonucleic acid of rabies virus. J. of Virology. 8: 922-924, 1971.

2. Hiatt, C.W., Shelokov, A. Rosenthal, E., and Galimore, J. Treatment of controlled pore glass with polyethylene oxide to prevent adsorption of rabies virus. J. of Chromatography. 56: 362-364, 1971.

3. Mitchell, J.R., Everest, R.E., and Anderson, G.R. Sensitive procedure for detecting residual viable virus in inactivated rabies vaccine. Applied Microbiology 22: 600-603, 1971.

EXTRAMURAL PROGRAMS - NIAID

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ANNUAL REPORT OF PROGRAM ACTIVITIES

National Institute of Allergy and Infectious Diseases
July 1971 - June 1972

I. REPORT OF THE ASSOCIATE DIRECTOR

In FY 1972, the Extramural Programs of the National Institute of Allergy and Infectious Diseases instituted a vigorous review of its program management through a much more extensive use of its program analysis capabilities, and through a more meaningful involvement of its National Advisory Council in the allocation of Institute resources by the Dollar Allocation by Program Area (DAPA) described below.

Several new program initiatives were also begun. They were Basic Biology of Venereal Disease, Lymphocyte Biology, Mechanisms of Immunity to Infectious Agents, and Cell-Mediated versus Humoral Antibody Responses. Staff and consultant reviews of proposed programs in Clinical Immunology and Diagnostic Virology were begun.

Research Grants

The FY 1972 regular research grant budget was increased 9½ percent over the FY 1971 level, enabling the Extramural Programs to support 65 more approved high priority applications than in FY 1971. However, only about 47 percent of the applications reviewed and approved by the National Advisory Allergy and Infectious Diseases Council for FY 1972 support were funded. The funding percentage of approved FY 1972 applications was similar to FY 1971 as a result of a 16 percent increase over the FY 1971 level of approved applications, a decrease in normal savings from grant budgets, cessation of staff-negotiated percentage reductions in grant budgets, and steadily increasing direct and indirect cost requirements.

Training Grants and Fellowships

While funds available to award approved training grant and fellowship applications in FY 1972 were unchanged from FY 1971, the number of approved fellowship requests increased 7 percent and the number of approved training applications dropped 13 percent. Thirty-seven percent of approved training grant applications were funded in FY 1972 by this Institute, the same percentage as in FY 1971. Only 42 percent of approved fellowship applications were funded in FY 1972 compared to 50 percent in FY 1971. The number of fellowship applications has been increasing for several years. 245 fellowship applications were reviewed in 1972 compared to 219 in FY 1971 and 149 in FY 1970.

Extramural staff continued its strict review of training grant budgets in accordance with Training Grant Committee and Council recommendations. The Training Grant Committee reaffirmed its policy of supporting senior personnel on training grants; i.e., reasonable salary support is appropriate for faculty members on training grants in an amount set by Committee recommendation and negotiated with the program director.

Criteria for approval of training grants in the fields of public health and medical microbiology were studied in detail by the Training Grant Committee, which concluded that the support of training grants in this area should continue to be evaluated on the research training component.

Dollar Allocation by Program Area and Special Emphasis Programs

During FY 1972, the Extramural Programs implemented the Dollar Allocation Program, following an in depth Institute-sponsored review of its Special Emphasis Programs. The definitions and specific goals of these programs were refined by staff and recommended for adoption by the National Advisory Allergy and Infectious Diseases Council. Funds were then allocated by Council to insure support of certain applications within the re-defined Special Emphasis Programs which would otherwise not be awarded due to limited fiscal resources. The new system enabled Council to devote more time to review of broad programs important to the health needs of the country rather than on review of individual projects within these programs.

The areas selected for Special Emphasis by the Extramural Programs were: Viral Hepatitis; Chronic and Degenerative Diseases of Man; Interferon; Mechanisms of Drug Resistance; Streptococcal Disease and Sequelae; Basic Biology of Venereal Diseases; Biological Regulation of Vectors; Immunity to Animal Parasites; Lymphocyte Biology; Mechanisms of Immunity to Infectious Agents; and Cell-Mediated versus Humoral Antibody Responses. A ceiling amount of $1,600,000 was allocated to nine of the eleven Special Emphasis Programs for preferential funding. Approximately 17 percent of the total financial resources available for research grants in FY 1972 supported projects within the areas of Special Emphasis.

Scientists were encouraged to undertake research projects in areas of Special Emphasis by Extramural Programs support of symposia, workshops, articles in journals, and staff contact with national scientific societies.

Program Analysis Activities

During FY 1972, more effective use was made of our Extramural Data Control and program analysis capabilities. In response to a question raised by our Council, an analysis was made of the extent to which our Institute uses the various mechanisms for program and center awards, and how this compares with the experience of other NIH Institutes. The analysis indicated that, of all the NIH Institutes, NIAID allocates the lowest proportion of its research grant dollars to program project and center awards. A report was given to our Council of a comprehensive analysis of the proportion of our research grants and training grants which involve clinical research components. The information contained in these and other program analysis reports provides a solid basis for staff to develop proposals for Council consideration regarding questions of program balance and important unmet needs.

Program Balance

An analysis was made of shifts in the numbers and dollar amounts of research grants assigned to each of the five NIAID Extramural Program branches over

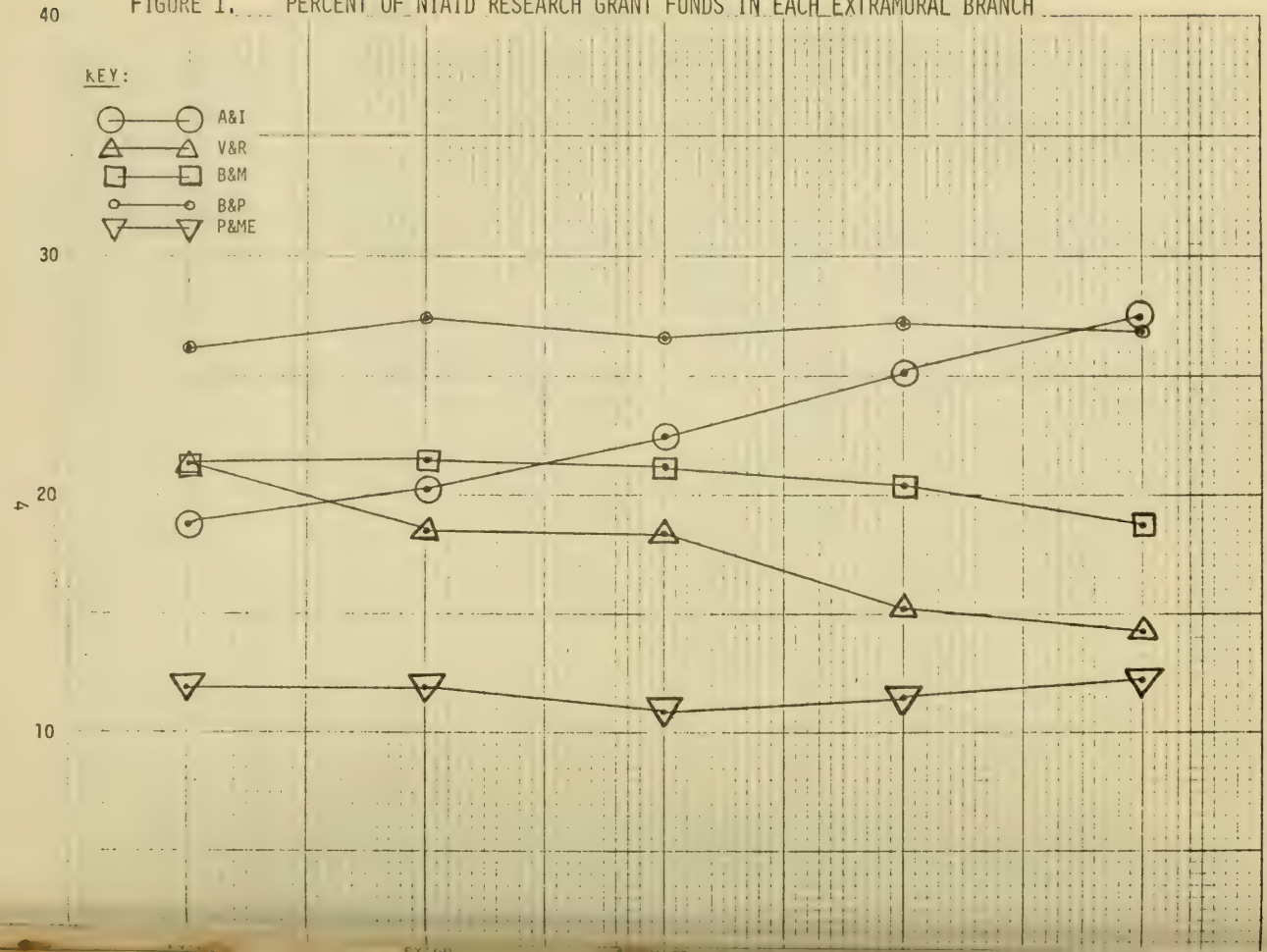
a period extending from Fiscal Years 1967 through 1971. Figure 1 summarizes the trends that were observed. This study revealed that some dramatic changes occurred during this period. The NIAID research grant budget was rather stable during this period, increasing about 5 percent from FY 1967 to FY 1971. The total number of research grants awarded decreased by 233 (17 percent) during this period, so that the size of the average NIAID research grant increased 26 percent, from about $33,700 to about $42,500. In every branch except Allergy and Immunology, the absolute number of research grants decreased. For the Allergy and Immunology Branch, the number of research grants increased by 40 (17.9 percent) which represented a 45.5 percent increase in the Institute's investment in Allergy and Immunology research grants. The number assigned to the Bacteriology and Mycology Branch decreased by 80 (23.4 percent), and the percent of total grant funds decreased by 12.6 percent. The sharpest branch decrease occurred in the Virology and Rickettsiology Branch, for which the decrease of 77 grants represented a 35.6 percent program decrease, and the decrease in dollar total of $3.144 million represented a lowering by 34.4 percent of the portion of total Institute research grant funds in this Branch. The situation regarding the Parasitology and Medical Entomology Branch was rather stable during this period. While the number of grants awarded decreased by 36 (17.8 percent), the dollar total increased 5.7 percent, and the percent of total NIAID re-research grant funds for this Branch changed less than one percent. While the number of research grants assigned to the Biochemistry and Physiology Branch decreased by 83 (21.0 percent), the total for research grant funds increased 10.3 percent, and the percent of total Institute research grant funds increased slightly, to 27.3 percent.

The increase of 51.8 percent ($4.567 million), during the FY 1967-1971 period, in the funding of research grants assigned to the Allergy and Immunology Branch reflects impressive advances that have been made in the field of immunology in recent years, leading to increased numbers of strong applications with payable priority scores. Since many of the more basic projects involving bacteriology and virology tend to be assigned to NIAID's extramural Biochemistry and Physiology Branch, it is not feasible to make sharp distinctions regarding trends in the virology and bacteriology areas. Thus, for example, a total of 176 noncompeting and competing research grant awards (for a total of $8.306 million, or 17 percent of NIAID research grant funds) were made in FY 1971 for research grants which had received initial scientific review from the Virology Study Section. For this same fiscal year, only 139 research grant awards were assigned to the Virology and Rickettsiology Branch ($6.881 million, or 14.1 percent of NIAID research grant funds).

Clinical Programs

A study was made of the clinical research projects and clinical research training grants currently being supported by the NIAID Extramural Programs. Of the 1,216 total research grants, 154 (12.7 percent) were clinical; i.e., studies founded in whole or in part on actual observation of patients, as opposed to purely basic research. The Allergy and Immunology Branch had the highest percent (27.0 percent) of clinical research grants, 75 of its 278 active grants being clinical. The average amount per clinical research grant ($55,600) was higher than that for total research grants ($42,000).

FIGURE 1. PERCENT OF NIAID RESEARCH GRANT FUNDS IN EACH EXTRAMURAL BRANCH



In contrast of the 178 active research training grants, 63 (35.4 percent) had a clinical component, and the bulk of these were concentrated in the Bacteriology and Mycology and the Allergy and Immunology Branches. Clinical research training grants were those in which about 50 percent of the training was concerned with clinical rounds, consultations, attendance at clinics, etc., and the research project may or may not be clinical.

Compared to the other nine institutes of the NIH, NIAID ranked third lowest in the percent of clinical programs among the active regular research grants (R01's) in FY 1971, the average being 23 percent. Institutes in which clinical programs consistently exceeded 20 percent since 1967 were NCI, NHLI, NIAMD and NICHD.

At their March 1972 meeting, the National Advisory Allergy and Infectious Diseases Council discussed program areas in which more clinical research could be encouraged, not only in order to attain program balance, but because of increasing prevalence and growing social concern about selected diseases. Among these diseases were venereal disease, fungal and other opportunistic infections, and intra-uterine infections (cytomegalovirus inclusion disease and toxoplasmosis).

The Allergic Disease Centers program, established in 1971, was expanded. A workshop of Allergic Disease Center program directors met in Bethesda in December 1971 to discuss progress in the application of new knowledge gained from recent advances in immunological science, including the clinical treatment and prevention of allergic disorders. In addition, program directors gained new familiarity with research activities of other centers within the program.

Personnel Changes

Dr. J. Allen Scott, Chief, Parasitology and Medical Entomology Branch, retired May 31, 1972.

National Advisory Allergy and Infectious Diseases Council

Members of the Council for the period ending June 30, 1972 were:

Dr. Elisha Atkins
School of Medicine
Yale University

Dr. Wilford S. Bailey
Auburn University

Mrs. Marian W. Bell
Pittsburgh, Pennsylvania

Dr. J. Werner Braun
Rutgers, The State University

Dr. Charles A. Janeway
Children's Hospital Medical Center

Dr. Philip S. Norman
Johns Hopkins School of Medicine

Dr. Thomas F. Paine
Nashville General Hospital

Dr. Everett R. Rhoades
University of Oklahoma

Dr. Frank A. Sloan
University of Florida

Dr. Carlos N. Vicens
City of San Juan, Puerto Rico

Dr. Duard L. Walker
The University of Wisconsin

Mr. William H. White
Family Health Magazine

Ex Officio Members:

Dr. James H. Matthews
Veterans Administration

Capt. Charles H. Miller
Great Lakes Naval Hospital

II. PROGRAM REVIEWS

A. ALLERGY AND IMMUNOLOGY

The approximate level of support given allergy and immunology:

Type of Grant	Number	Amount
Research grants	324	$15,013,000
Training grants	48	2,855,000
Fellowships	68	1,181,000

Summary

From the relatively minor status of a branch of microbiology directed toward an understanding of the defense mechanism of the body and the practical application of this knowledge to infectious disease immunoprophylaxis, immunology has progressively undergone a transformation in problem formulation and in scope. In the course of the last two decades, the immune response has come to be recognized as a subtly balanced response to fluctuations in the internal macromolecular environment. The objectives of immunology have gradually been transformed within the framework of molecular biology. Impressive advances have been made in unravelling the complex cellular interactions which underlie the immune response, thus beginning to attain an understanding of the area that is broadly described as "regulation."

It has become clear that the immune system is essentially equivalent to the host's total population of lymphoid cells. The central object of basic immunologic investigations are these lymphoid cells and the antibodies and other immunologically distinctive molecules they elaborate. All natural organic substances as well as an unlimited number of synthetic organic, and even non-organic compounds, can induce the formation of specific antibodies when introduced parenterally. Antibodies combine with the antigenic agents that induced their synthesis; i.e., they "recognize" their corresponding antigens. The number of antigens in man's environment is large indeed, and the individual has the capability to respond to most of these stimuli and to synthesize an enormous number of different antibodies. This diversity has been and continues to be a source of fascination for immunologists seeking to understand the generating and the regulating mechanisms involved. It would appear that each of the approximately 10^{12} lymphocytes in an adult is restricted to producing antibodies of one specificity; i.e., antibodies which react specifically with only one antigen and that each cell possesses the structural genes necessary for this task. Among the central issues in immunology, two continue in the forefront: (1) how the lymphocyte population of an animal acquires this extraordinary diversity of genes, and (2) how the cells register an antigenic signal, and in which ways the entire system, with its almost infinite capacity to respond, is kept under regulatory control.

This past year, immunology has continued to grow and expand, in an exponential fashion, in its impact in biological science. However, even in this impressive growth context and continuation of significant advances on all fronts, this period has seen several especially notable developments, of a kind that seem

destined to have a profound and enduring impact on the character of the field. Briefly, these developments are: (a) the cell membrane of lymphocytes is proving to be distinctive in being fluid phase in character; this has special implications with regard to the reactions of receptors on bone-marrow and thymus-derived lymphocytes; (b) there is occurring a radical restructuring of our understanding of immunological tolerance. The historic concept of specifically unresponsive cells is being abandoned in favor of active control, i.e., immune lymphocytes are in fact present, but their activity and response to antigen is held in check by blocking or other antibodies; (c) causal relationships are being uncovered between histocompatibility antigens, immune responsiveness and susceptibility to disease; and (d) there has evolved an awareness of the subtleties of cell-cell interactions and the consequent elaboration of both specific and non-specific signals; this work is finally establishing the framework for understanding immune recognition and regulation at the molecular level.

ALLERGIC DISEASES CENTERS PROGRAM

The past year saw this program actually begin operation in the form of seven centers that were funded beginning September 1971. In December, the program directors and senior staff of each of these centers were invited to Bethesda for the purpose of exchanging information on the orientation of their respective research programs and progress to date. Good internal communication amongst the seven groups was thus established, a community of interests was identified, and various concerns, both technical and conceptual, common to the group, were reviewed. The success of the meeting assured its continuation under NIAID support as an annual function.

A second group of 11 new applications and three supplemental requests from the seven present centers was received early in 1972 and reviewed in April, by a special study section. Two of the new applications were approved; thus, this program will be modestly expanded during this year.

LYMPHOCYTE BIOLOGY PROGRAM

This program seeks to attain the potential implicit in the emerging technology of short- and long-term culture of lymphocytes in vitro as the principal means for eventual utilization in immunology of the impressive approaches and techniques heretofore unique to microbial genetics. The program previously elaborated by the Allergy and Immunology Branch was accepted by the NIAID Council as a Special Emphasis Program. It was successfully launched at the Albert Einstein College of Medicine, with the activation of the first of a series of program projects.

Extensive exploratory discussions were subsequently held with national experts at Stanford Medical School, at the Rockefeller University, and at Yale University. These Institutions are among the relative handful that have, in each instance, a critical nucleus of outstanding talent in the disciplines especially relevant to the cell biology of lymphocytes. The fields primarily involved are: somatic cell genetics, cell biology, hematology, tissue culture and various immunology specialties. Few institutions have the requisite experienced principal investigators in each of these disciplines such as is required to

evolve this kind of program. Each of the aforementioned groups is presently engaged in formulating a multi-disciplinary program project that would, in its own distinctive fashion, advance the concepts defined in the Council-approved program description. Applications for review will therefore be arriving later this year.

CLINICAL IMMUNOLOGY PROGRAM

From an extended series of discussions and consultations with eight ad hoc national experts, a position paper was prepared which identified (a) the character of the field at present; (b) its importance nationally; (c) the need and opportunities; and (d) the distinctive approach to dealing with immunologic disease via the creation of Clinical Immunology Units at major centers of academic medicine. This document, identifying the means for enhancing the development of clinical immunology, provided the focus for a series of discussions by NIAID senior staff.

As envisioned in the program, the clinical immunology unit orientation in a medical center would be distinctive in the following respect: the professional staff would be individuals experienced in basic immunology and fully competent as clinical investigators. It would have its own research program in one or another of the immunological subspecialties relevant to clinical immunology, the quality of which would be a major determinant in the evaluation of the unit for such an award. In addition to their basic research program, the unit would be oriented toward the application of basic immunologic knowledge to the diagnosis, management and therapy of immunologic diseases and derangements.

The thrust of this program is to give greater visibility to the fact that immunologic disorders are far more frequent and widespread than is generally realized, and to demonstrate how properly trained and oriented groups committed solely to this task, can cope effectively with this challenge. Finally, the NIAID as the principal funding Institute for the discipline of immunology seeks in this program to lead in the advancement of immunology as regards utilization of its basic knowledge for better delivery of health care.

In October, the Chief, Allergy and Immunology Branch, served as a temporary advisor to the WHO immunology unit and participated in the meeting in Geneva, Switzerland, of an international group of experts in Clinical Immunology. In the course of a week's discussion, this group drafted an official WHO position paper on organization of Clinical Immunology Units and their function, which in fact enbodies a number of the key proposals in the aforementioned Institute position paper. These documents were presented to NIAID Council and served as the basis of an extended discussion on the merits of an Institute program in Clinical Immunology. The subject was considered at the March Council, but action was deferred pending Institute review of its total support in the field of immunology.

PERSPECTIVES IN IMMUNOLOGY - THE BROOK LODGE CONFERENCES

The fourth volume in this series, representing the scientific product of last year's conference on "Immunologic Intervention" was published by Academic Press

in March 1972. It is presently being reviewed by Professor Alain De Weck of Berne, Switzerland, and Dr. Fred Rosen of Boston, eminent clinical immunologists. The volume also represented the basis for a feature story in the New York Times on the application of immunologic knowledge to clinical medicine.

The fifth Brook Lodge Conference on "Genetic Control of Immune Responsiveness in Relation to Disease Susceptibility" was held May 8-10, 1972, and, in contrast to the previous conferences, had to deal simultaneously with a flood of new data and findings, as well as overall judgment and assessment of the position and prospects in this most compelling newly emerging aspect of immune regulation. The major issues taken up included: (1) the histocompatibility-linked immune response genes with regard to their allotype locus of action and the distinctive organization and relationship with histocompatibility genes; (2) immune response genes linked to immunoglobulin allotype and other types of immune response genes; (3) relationship between histocompatibility-linked and allotype-linked immune response genes and antigen-specific receptors on T and B cells; (4) an exploration of the data on multigenic control of disease susceptibility yielded by animal models; and (5) a critical assessment of the association between HL-A type and specific disease entities.

These developments portend the final emergence of an understanding of how and why differences occur in the immune response to various antigenic models, as evidenced in different species, within a species in genetically defined lines and, in its most subtle form, among individuals of a species even when genetically similar - but not identical. Implicit in this amorphous, pervasive area is the certainty that within a relatively short time, it will be possible, by typing host lymphocytes for their profile of "H" and other receptors to make far reaching predictions concerning the probability of subsequent development of various disease entities. Thus, both in a qualitative and qualtitative dimension, immune responsiveness is now perceived as a highly individual trait in an outbred population such as the human. The edited version of these proceedings will be published in November 1972.

With completion of the projected cycle of five conferences and the publication of the volumes which represent their scientific product, both are viewed as constituting an unique element in the contemporary immunologic scene. The five-year span in which they were held has seen a plethora of conferences devoted to both general and highly specialized subject. However, this series clearly stands apart from other meetings in its almost total orientation towards interpreting, evaluating and projecting future directions in areas that constitute the leading edge of contemporary immunology. The published volumes "Perspective in Immunology" have achieved general acceptance as a distinctive and valuable contribution to the immunologic literature. They have also contributed to the projection of a purposeful public image of this Institute's commitment to the discipline of immunology.

AI BRANCH PROGRAMMING

During this period, initiatives were concerned with arranging for research projects by outstanding experts on subjects viewed as especially timely and

opportunistic and of unusual import for the Special Emphasis Programs of this Branch. Particular emphasis was given the planning of conferences that contribute to the furtherance of the discipline of immunology.

Research projects on the following subjects were programmed:

1. Aging and age-related diseases as expressions of malfunction of immunologic control mechanisms.

2. Further physico-chemical characterization of the soluble mediators elaborated in vitro by mitogen-activated lymphocytes.

3. Factors involved in the establishment of human lymphocytic cell lines.

4. Augmentation of tumor immunity by components derived from Mycobacteria.

Meetings organized:

1. Program plans for the seventh Leucocyte Culture Conference held in June 1972 were made based on diminished emphasis on research reports and more to review, evaluation, and identification of new directions in lymphocyte biology.

2. A workshop was organized with 12 international experts in cellular immunity and held at Rockefeller University May 12-13 to ascertain problems and progress in the clinical utilization of the distinctive in vitro methods of this specialty.

3. The planning since 1970 of an international conference on "Non-Specific Factors Influencing Host Resistance," finally culminated in the meeting held in Berne, Switzerland, June 19-21. The Chief, Allergy and Immunology Branch, did much of the organization and participated in the conference.

RESEARCH GRANT HIGHLIGHTS

AI-09807-03 - B. R. Bloom

A virus plaque assay has been developed for enumerating antigen-sensitive cells in delayed-type hypersensitivity. When lymphocytes from delayed hypersensitive individuals were stimulated by specific antigen under culture conditions known to produce mediators, the cells became capable of replicating two different RNA viruses. Infected cells sampled and plated in agar above a monolayer of indicator cells susceptible to the virus make possible enumeration of the cells that have been activated by antigen to produce sufficient virus to cause a plaque in the indicator monolayer. The number of plaques is a function solely of the lymphocytes, and indifferent to the virus employed. In the case of guinea pigs exquisitely sensitized to tuberculin by Freund's adjuvant, it was possible to detect approximately 1.6 percent of the lymph

node cells after PPD stimulation reactive in this assay. In contrast, when human peripheral lymphocytes from first test strength positive tuberculin hypersensitive donors were used, the maximum number of cells which could be stimulated by antigen with virus plaques was found to be approximately 5/1000 peripheral blood cells. Control cells taken from individuals producing only circulating antibody, not known to be hypersensitive, have shown no stimulation by antigen in the virus plaque assay. Thus, this assay correlates with cell-mediated reaction in vivo; it may well provide the only index presently available of the number of effector cells.

As regard the type of cells being measured by this assay, the majority appear to be large lymphocytes, with organized polyribosomes in the cytoplasm, but without the morphological differentiation either of blast cells or of secretory cells. They would be classified as medium or large lymphocytes.

On the basis of tests with thymus specific serum, the origin of the virus plaque forming cells was investigated. Such serum, under appropriate conditions, kill approximately 80 percent of the virus plaque forming cells, and when the surviving 20 percent are reconstituted to the original cell concentration, the normal percentage of antigen-sensitive cells is restored. Thus, this serum selectively kills only T cells. These and other experimental approaches are consistent with the view that this assay is dealing primarily with T cells.

AI-10158-02 - M. L. Howe

The isogeneic lymphocyte interaction (ILI) is characterized by proliferation of neonatal murine thymocytes which have been incubated in mixed culture with isogeneic spleen cells from adult mice. This reaction offers a means for developing insight into the role of the thymus in the ontogenesis of the mammalian immune system.

Thymus cells of newborn mice (1-2 days of age) were found to respond vigorously in the MLC and ILI and to mitogen. Responsiveness of thymocytes in all of these reactions was found to decline with age. The age dependent decrease in reactivity of thymocytes in the MLC and to mitogen appeared to correlate inversely with the increasing reactivity of spleen cells of older animals. In contrast, PHA responsiveness was found to be lacking in both neonatal and adult thymocytes.

It was possible, however, to detect PHA reactive cells in the adult thymus 24 hours after in vivo administration of cortisone acetate, a procedure which is known to selectively deplete the thymus of immature cells. Concomitant increases in the reactivity of thymocytes from cortisone treated donors to mitogen and in the MLC were also observed, but no ILI reactivity was detectable in the adult thymus before or after cortisone treatment.

Although thymocytes from normal neonatal mice exhibit considerable reactivity to mitogen and in the MLC and ILI, it was found that cortisone treatment of neonates resulted in dramatic increases in all of these activities. Interestingly, neonatal thymocytes failed to respond to PHA, even after cortisone treatment.

Thus, during ontogenesis, an inverse relationship exists between the capacity of thymocytes to respond in the ILI and the capacity of spleen cells to stimulate in this reaction. In the neonate, when thymocyte ILI responsiveness is greatest, the capacity of spleen cells to stimulate is much reduced. Conversely, in the adult when the ability of spleen cells to stimulate is maximal, ILI responsive cells cannot be detected in the thymus even after cortisone treatment.

AI-10155-02 - P. A. Ward

Substances released in vitro by sensitized lymphocytes in contact with specific antigen are thought to be the mediators of delayed hypersensitivity (DH) in vivo, but evidence on this point is indirect: activated lymphocyte culture supernates may produce inflammatory reactions in guinea pig skin. Also, such supernates can mediate the macrophage disappearance reaction (an in vivo correlate of DH). In this study, various skin reaction sites were homogenized into Medium 199 with a Virtis blender and the extracts were centrifuged free of particulate matter. Supernates from DH sites were found to increase the macrophage content of peritoneal exudates in guinea pigs that had previously received i.p. glycogen. These supernates also showed in vitro chemotactic activity for macrophages and lymphocytes. They were not capable of mediating the migration inhibition reaction. In contrast, supernates from Arthus sites had neutrophil chemotatic activity and were otherwise negative in the various studies performed. Antigen or glycogen-injected skin of normal animals yielded extracts that were uniformly negative.

Supernates from DH sites produced mononuclear infiltrates in skin of unimmunized animals; controls did not. These results show that active soluble factors are indeed present at DH sites and suggest that these factors, especially those with chemotactic activity, may play a role in the evolution of these reactions.

AI-06552-08 - J. W. Jutila

Congenitally thymusless mice (nude) reared under specific pathogen-free conditions developed classic symptoms and many died of wasting disease at 15 to 24 weeks of age. Nude mice were found to have lowered lymphocyte and elevated neutrophil counts when compared to their phenotypically-normal littermates. Although serum levels of IgG_2 and IgM were normal in nudes, they made less IgG_1 and significantly less IgA than their normal littermates. Histological examination of the lymph nodes and spleens of nudes showed severe lymphocytic deficiency in the thymus-dependent areas. Infections were commonly detected in organs of older nudes. Assays for fecal immunoglobulins revealed a paucity or complete lack of IgA in the feces of nudes suggesting that the secretory immune mechanism of the gut was seriously impaired. Significantly, as IgA disappeared from the gut of nudes, massive numbers of coliforms developed in the ileum, ceacum and large bowel, an event rarely observed in normal control mice. IgA acquired through coprophagia may explain the observation that nudes reared with normal littermates were healthier and survived longer than nudes reared alone. The evidence suggests that a consequence of the congenital athymic condition is an impairment of the secretory immune mechanism which, in turn, predisposes to infections by normal flora.

AI-06754-08 - F. Milgrom

IgM agglutinins against human cultured lymphoid cells were demonstrated by means of a slide agglutination test in 21 of 64 infectious mononucleosis (IM) sera, five of 19 SLE sera, but only in one of 64 normal sera. Positive IM sera reacted with all four lymphoid cell lines employed. Absorption of the serum with one cell line removed its activity against all cell lines. No correlation was found between the lympho-agglutinins and NDV-O agglutinins, anti-EBV antibodies or lymphocytotoxins in the IM sera. The lympho-agglutinins were also shown to be different from the heterophile antibodies since absorption of an IM serum with sheep or bovine erythrocyte stromata did not affect its lympho-agglutinating activity. However, absorption of the serum with lymphoid cell cultures removed not only the lympho-agglutinins but also the heterophile antibodies. It was also shown that normal human peripheral leukoxytes, erythrocytes and epithelial cell lines carry neither the antigen for the agglutinins nor the heterophile antigen. On the basis of these results, cultured lymphoic cells seem to acquire these two antigens during propogation. It appears also plausible that the patient's own transformed lymphocytes may acquire similar antigens in the course of IM, which would account for formation of the lympho-agglutinins and the heterophile antibodies.

AI-09419-09 - E. Thomas

Cellular immunity to host antigens and serum blocking factor are present in canine long-term chimeras. Marrow grafts were carried out between sibling donor-recipient pairs selected by serological histocompatibility typing and mixed leucocyte culture in a manner similar to that used to define human HL-A matched siblings. To define the nature of the graft-host "tolerance" in histocompatible pairs, six canine marrow chimeras and their living donors were studied using a modification of the colony inhibition assay. Donor or recipient lymphocytes were added to each of 8-12 wells containing either donor or recipient skin fibroblasts which had been pretreated with either donor or recipient serum. After three days the remaining fibroblasts were counted. Each pair was tested on two separate occasions using fresh or frozen lymphocytes. In five or six animals, chimeric lymphocytes significantly inhibited ($p.0.05$) the growth of chimeric fibroblasts. The inhibition ranged from 12-44 percent. In each case, chimeric serum blocked the inhibitory effect of chimeric lymphocytes. The data suggest that even in histocompatible chimeras, the "tolerant" state is an active process. Cellular immunity in these animals must be directed to "minor" histocompatibility antigens not detected by current typing methods.

AI-10180-02 - R. A. Reisfeld

Rabbits immunized with soluble HL-A antigens isolated from cultured human lymphoid cells by 3M KCl extraction produced precipitating and/or cytotoxic antibodies. The precipitating antibody was detectable only in the sera of animals injected with soluble HL-A antigens in complete Freund's adjuvant. The antibody did not correlate with HL-A specificity, but cross-reacted with soluble murine $H\text{-}2^d$ antigens and judged from absorption data, seemed directed against antigen(s) not readily available on the cell surface. However, cytotoxic antibodies with titers from 1:2 to 1:512 were produced by rabbits immunized

with soluble HL-A antigens either with or without adjuvant. The specificity of the heteroantisera was evaluated against a cell panel of at least 80 donors and compared to that of 115 well-characterized alloantisera recognizing 25 HL-A specificities. A close correlation was observed with the HL-A determinants of the immunogen. Some diluted rabbit sera appeared monospecific and their use as typing reagents is under investigation. The heterologous antisera did not stimulate DNA synthesis in human leukocytes suggesting that these antisera contained few antibodies directed against surface components effecting blast transformation. In indirect immunofluorescence assays, the heteroantisera revealed a "patch-like" distribution of HL-A determinants of the soluble immunogen.

AI-09114-04 - S. Cohen

When stimulated by specific antigen, sensitized lymphocytes secrete soluble mediators such as migration inhibition factor, chemotactic factor, and substances which enhance phagocytosis. Interfacial differences at cell surfaces appear to play a crucial role in phagocytosis. The effect of lymphocyte culture supernatants on the angle a drop of saline placed on a macrophage monolayer makes with that monolayer was determined. This contact angle is a measure of the interfacial tension between the cell surface and the fluid medium. Lymphocytes from delayed hypersensitive guinea pigs were cultured in the presence or absence of antigen according to standard techniques for generating mediators. These supernatants were incubated with guinea pig alveolar macrophages for one hour at 37°C. The antigen-activated supernatants reduced the contact angle significantly and were comparable to the change produced by the activation of macrophages by the ingestion of hydrophobic particulate matter. Neither antibody nor antigen had a similar effect, nor did the supernatants have detectable cytophilic antibody.

AI-08677-18 R. A. Good

Phytohemagglutinin (PHA) stimulation of lymphocytes has been used to assess overall function of thymus-derived, cell-mediated immunity ("T"-cell function). Lymphocytes, a morphological entity, comprise five broad functional subgroups, i.e., 1) thymus-derived (T) cells; 2) bursa-derived (b) cells; 3) monocytes; 4) partially differentiated stem cells; 5) primitive stem cells. The conventional method of isolating lymphocytes invariably results in random selection of these subgroups. Therefore, the evaluation of "T" cell function using isolated lymphocytes does not provide quantitative analysis. A new micro-method of PHA stimulation of lymphocytes was developed using direct, unfractionated whole blood culture technique. It was shown that the vigor of lymphocyte response to PHA stimulation is directly proportional to the amount of incorporated H^3TdR in the lymphocytes in the unit volume of blood. Hence, the quantitation of "T" cell function is possible. A large scale clinical survey provides a precise quantitative evaluation of "T" cell function in a variety of clinical diseases. This method was extended to human marrow. The results disclosed that relatively pure marrow cells do not respond to PHA stimulation unless contamination of peripheral blood has taken place during aspiration.

AI-10153-02 - E. J. Yunis

The relationship between phytohemagglutinin (PHA) response and incidence of autoantibodies in an aging human population is being investigated. It has been shown in mice that decline of cell-mediated immunity and decreased PHA response is associated with a high incidence of autoantibodies and low survival. In this work, the PHA response in 137 Caucasians aged 60-101 years, and 48 normal controls aged 20-35 years, was measured. Antibodies against thyroglobulin, nuclear antigens and rheumatoid factor were determined. The mean PHA response of the normal controls was 132,977 cpm, whereas responses in the aged group were less than 71,141. The incidence of autoantibodies increased progressively with age. There were 20 individuals with autoantibodies among the 99 who had normal PHA responses, and 19 in the 38 persons with decreased PHA responses. The association between low PHA response and autoantibodies in aging humans is consistent with similar findings in aging autoimmune susceptible strains of mice and thus further supports the concept that immune deficiency is important in the diseases of aging.

B. BACTERIOLOGY AND MYCOLOGY

The approximate level of support given bacteriology and mycology:

Type of Grant	Number	Amount
Research grants	223	$10,189,000
Training grants	46	2,588,000
Fellowships	52	894,000

Summary

This Branch continued to support a high quality program of research covering areas of infectious disease caused by bacteria and fungi. Activity in these areas has been continued at a consistently high level, and research progress in some of these has been unusually productive. Support has continued to decline for research in areas considered to be of marginal importance to human health problems, such as studies on microorganisms involved in plant pathogenesis.

The two Special Emphasis Programs of the Bacteriology and Mycology Branch on Streptococcal Diseases and Sequelae and on Drug Resistance were redefined and approved by the National Advisory Allergy and Infectious Diseases Council as programs with a more narrowly focused research emphasis. The Streptococcal program now puts major emphasis in two areas, one on the Biology of the Streptococci and the other on Pathogenesis and Clinical Aspects. The Drug Resistance program, now known by its new title of Mechanisms of Drug Resistance, emphasizes the genetic mechanisms involved in the development of resistance of microorganisms to antibiotics. Both special programs deal with fundamental aspects of two current problem areas of widespread importance.

A third Special Emphasis Program, on the Basic Biology of Venereal Diseases, represents a newer and broadened initiative by the NIAID to focus on and enlarge the areas in our knowledge relating to the responsible microorganisms and the pathogenesis of venereal diseases. This Special Emphasis Program is an outgrowth of two workshops organized by the Extramural Programs of the Institute. The first one explored the problem areas of the gonococcus and the current widespread epidemic of gonorrhea. The second explored the most important aspects of syphilis and of the treponemal causative agent. Results of both workshops have now been published in the Journal of Infectious Diseases, the first one appearing in October of 1971 and the second in March of 1972. Response to this new program initiative has been very gratifying; the program is currently funded at a level of $456,000 with new applications for research in venereal disease continually being received for scientific review. Staff efforts to increase awareness in the scientific community of the research needs in venereal disease are continuing.

In addition to the three special programs described, other fields of research in the microbiology of important diseases have maintained the interest of many capable workers. Promising developments have been described in tuberculosis therapy, in leprosy and cholera research, in bacterial endotoxin mechanisms, and in the ever-important area of fungal or mycotic infections. The latter continues to be a problem in great concern as a major hazard to many patients

with impaired immune defenses; it is one of the leading causes of morbidity and mortality in such patients.

RESEARCH GRANT HIGHLIGHTS

A number of noteworthy research fundings by grantees of the Bacteriology and Mycology Branch are presented here in brief form:

Mechanisms of Drug Resistance

AI-07715-05 - S. Cohen

This very productive worker has been studying the transferable R factors (the resistance factor) in the development of drug resistance in bacteria. He has found transferable R factors of gentamicin in E. coli strains, as well as transferable factors for multiple antibiotic resistance. The studies also suggest a slow increase in the prevalence of gentamicin-resistant Gram negative bacilli. Furthermore, hospital strains of Ps. aeruginosa showing resistance to carbenicillin and gentamicin are becoming more prevalent. This situation may restrict the range of useful antibiotics for therapy of these infections.

AI-10108-01 - A. Braude

During surveillance of patients with serious bacteremias, the most frequent isolate was Klebsiella pneumoniae, with E. coli the second most frequent cause of bacteremia. Multiple antibiotic resistance was found in over 9 percent of the isolates. More importantly, 60 percent of the patients died from these bacteremias despite intensive antibiotic therapy. The use of specific antiserum, especially in E. coli experimental infections in animal models, lowered the mortality rate by over 75 percent. These results are encouraging for future therapy of such serious infections.

AI-09584-02 - W. McCabe

Dr. McCabe has found that cross-reactive shared antigens of Gram negative bacilli diminish the severity of bacteremia in experimental animals and in patients. This finding may have important implications for the control of this type of disease, although further evaluation is warranted.

AI-00658-16 - E. Neter

Preparations of the Common Antigen (CA) described by Kunin, found in numerous enterobacterial species, evoked antibodies which promoted phagocytosis of bacteria possessing this antigen on the cell surface. Dr. Neter has also found that isolated erythrocyte receptors bound the CA more effectively than antigens of Gram positive species. Immune responses were detected in patients with urinary tract and bladder infections. Additional studies underway show considerable promise of the CA for possible usefulness in immunization against a variety of Gram negative bacterial infections.

AI-10079-01 - C. Kunin

The antibiotic polymyxin was found to bind to the acid phospholipids of mammalian cells in a manner similar to its binding to bacterial membranes. This may explain the peculiar neuro- and nephrotoxic potential in man of this material. Persistence of the antibiotic was also found on tissue membranes containing lipid and acid phosphatides. This phenomenon may account for the cumulative dose-related toxicity long after the drug has disappeared from the serum. Colistimethate, similar structurally and in activity, remains free and active in the tissues without binding, because of the process of methane sulfonation. It is, therefore, less toxic and potentially more active.

AI-09838-01 - J. O. Lampen

The major fungal polyene antibiotics, such as candicidin and amphotericin B, have a major effect on red cells and yeast protoplasts by opening up holes, or pores of substantial size, on the cell membranes. Other polyene antibiotics, e.g., filipin and mediocidin, have high cellulolytic potencies by creating large diffusion pores on the fungal protoplast membrane.

No correlation between hemolytic potency and membrane binding efficiency was seen. The high hemolytic potency of mediocidin and filipin may reflect the fact that only very low levels of these antibiotics need be bound to cell membranes in order to cause lysis. For all the polyenes tested, the sequence of events leading to hemolysis in the absence of osmotic protection of the cells involved the same colloid-osmotic mechanism. Holes in protoplast membranes can be opened in organisms by suitable polyene antibiotics, and the ability of the altered membrane to carry out appropriate functions can now be studies.

Cholera

The major objectives of the cholera research programs are a delineation of the mechanisms by which cholera enterotoxin causes gut electrolyte loss, and a more definitive understanding of antitoxic immunity.

AI-07625-05 - C. L. Carpenter

In a dog ileal loop model for studying cholera toxin, it was demonstrated that the degree of protection against challenge with enterotoxin correlated significantly with the circulating level of antitoxin in immunized animals, or in animals continuously infused with hyperimmune plasma. No studies have yet been able to demonstrate any effective local intestinal immune mechanisms.

AI-08877-03 - R. A. Finkelstein

Cholera toxin, or choleragen, was prepared in purified form as a crystallized protein with characteristic toxic properties. In addition, a naturally occurring inactivated (toxoid) form of the toxin, possessing both diarrheagenic and skin reactive properties, was also discovered. This may be the first report of a naturally occurring bacterial toxoid. The cholera toxoid, called

choleragenoid, is formed from choleragen toxin by vigorous agitation or by heating, probably through an intermediate, non-toxic polymer stage. Significantly, the choleragenoid does not revert to the toxic stage - it may therefore prove very useful for immunization of man against cholera.

AI-10066-01 - R. A. Finkelstein

Dr. Finkelstein is also evaluating the potential role of enterotoxic substances elaborated by E. coli strains in a study on the pathogenesis of the diarrheal state. With the ligated ileal loop model in adult mice, he has found that E. coli strains isolated from infant diarrheas caused a significant fluid accumulation in the test mice. These toxigenic strains also caused hemagglutionation of chick erythrocytes. Attempts are currently underway to isolate and purify the toxin.

AI-10074-01 - B. R. Sack

Strains of E. coli from diarrhea patients were found to produce an enterotoxin. In a study using the rabbit ileal loop model for detection of toxin-producing strains, it was found that an immunologic similarity exists between enterotoxins of different E. coli toxinogenic strains. There is also an immunologic similarity between these toxins and the cholera enterotoxin.

AI-08209-04 - W. B. Greenough

In a study of cellular responses to cholera toxin, Dr. Greenough found that toxin increases the activity of the cell membrane-associated enzyme, adenyl cyclase. This in turn produces changes in the cellular concentration of cyclic AMP. This indicates that the secretory process of the small intestine is probably hormonally-related. The mechanism of these relationships, especially the toxin-cell binding, is not yet known. Suitable pharmacologic agents that can block the effects of the diarrhea-producing toxin interaction on cell membranes are now in prospect.

Studies on Intestinal Flora of the Human Gut

AI-07328-05 - R. Freter

The objective of this study is to understand the mechanisms by which the normal intestinal flora maintains its remarkable stability to prevent growth of enteric pathogens in the intestinal tract. Germfree animals maintained on a crude diet were not rendered intestinally normal after implantation of a mixed bacterial flora in their gut. Data gathered from experimental animals did not support the hypothesis that fatty acids elaborated by intestinal anaerobes inhibited pathogenic enterobacteria. It was found that E. coli populations were controlled by the predominant anaerobes, rather than by means of colicins or other inhibitors, as usually believed. This study may have practical application for the implantation and maintenance of optimal intestinal floras in patients with gastrointestinal disease.

AI-07587-05 - T. Kent

The role of enzymes liberated from intestinal bacteria, especially of glucuronidase, may have great implications for the metabolism of drugs and toxic compounds, since many of these drugs are excreted by the liver as glucuronide complexes. The bacterial breakdown of these complexes could result in rapid drug or toxin recirculation, since it is known that the breakdown occurs primarily in the colon where absorption is rather slow. The bacterial breakdown, when it does occur in the small intestine, is the result of an increased enzymatic activity. The greater absorption of materials in this situation could have serious implications regarding the re-absorption of drug or toxin breakdown complexes.

AI-08223-04 - L. Weinstein

Hospitalized patients were studied during a period of non-exposure to antibiotics. Major changes were found in the human fecal microflora of these patients during their hospital stay, ranging from normal E. coli to colonization by other Enterobacteriaceae. Increased carriage of Klebsiella species, Proteus, and Pseudomonas were also noteworthy. By contrast, normal apartment dwellers, as controls, showed no change in their fecal coliform populations. Confinement in a hospital, therefore, is associated with significant changes in the microflora of the human intestine, probably as the result of prolonged exposure to these organisms in the environment.

Endotoxins

Bacterial endotoxins play a major role in contributing to the morbidity and mortality of certain human diseases. Antimicrobial therapy, in general, has not been successful in the management of patients in shock due to Gram negative bacteria, so that search for other measures is still necessary.

AI-10353-01 (formerly AI-08835-03) - J. J. Corrigan, Jr.

Results of this research demonstrated that the antibiotic polymyxin B contains strong anti-endotoxin properties, as shown by the failure of test animals to develop the endotoxin shock syndrome when given the antibiotic and endotoxin. The mechanism of the endotoxin polymyxin B interaction is probably that of a cationic detergent. This study should also provide a better understanding of how endotoxin activates the blood coagulation mechanism.

AI-07257-05 - J. W. Shands

Pathological studies of endotoxin-treated mice showed that the liver was severely affected, with extensive sinusoidal damage, necrosis of hepatic cells, and the extensive release of enzymes from lysosomes and cellular cytoplasm. Further study also suggested that the genetic resistance to endotoxin may be due, at least in part, to host cells which are less reactive to endotoxic substances. Research into the mechanism of endotoxemia may have potential application to the problem of acute Gram negative bacterial infections in man.

AI-10087-01 - L. J. Berry

The effect of endotoxin on inducible hepatic enzymes has been studied intensively for some time by Dr. Berry and his group. Two enzymes have been purified from mouse liver, then used to develop anti-enzyme antibodies (immune globulins) in rabbits; these antibodies were purified by precipitation and fractionation. The two enzymes, both of which are found in the soluble fraction of liver homogenates, are phosphoenolpyruvate carboxykinase (PEPCK) and tryptophan oxygenase (TO). The anti-enzyme immune globulin has been used in determining whether endotoxin inhibits the induction of these enzymes by reducing their synthesis in the liver, or by releasing an inhibitory substance in vivo which binds to the enzyme or blocks its catalytic action. By a radial immunodiffusion technique, it was clearly demonstrated that endotoxin inhibits enzyme synthesis in a manner indistinguishable from that seen after liver poisoning with actinomycin D.

Venereal Diseases

Research efforts are intensifying in this new Special Emphasis Program, particularly in studies on the gonococcus. One of the most interesting of recent findings has been described recently by an NIAID-supported Fellow:

F3-AI-50,525-01 - T. Buchanan

Pili are filamentous structures present on the surface of virulent gonococci; no pili have been found on avirulent cells. Colonial variants T1 and T2 of the gonococcus, containing pili, produced experimental infections in human volunteers, unlike the T3 and T4 variants devoid of pili. These pili have been purified by immunochemical techniques, and used to develop an antigen binding test assay with radioiodine labeled material. Of great importance is the finding that antigen binding in patients with gonorrhea is significantly higher than in non-infected control patients. This test may prove very useful for diagnosing gonorrhea, especially in the detection of asymptomatic carriers.

Leprosy

Continued research in this disease is necessary. There are many cases of leprosy occurring throughout the world in areas of importance to the U.S. Furthermore, an increase in U.S. cases is predicted for the future based on the large numbers of U.S. military personnel exposed while on duty in Viet Nam, a highly endemic area for leprosy.

AI-07266-05 - C. Binford

Results of Dr. Binford's studies have been of value in increasing the usefulness of the mouse foot-pad technique in leprosy research. Shielding of the bone marrow of mice by lead strips during whole body irradiation caused enhancement of growth of M. leprae in the foot-pad. This immunosuppression technique could broaden the use of experimental leprosy in animals, and it is expected that important studies can now be performed in larger animals with longer life spans than the mouse.

One significant new finding of great promise is the study of the potential usefulness of the armadillo as an experiemntal animal model for leprosy:

AI-07890-05 - W. Kirchheimer

Dr. Kirchheimer has reported that armadillos in captivity, after infection with virulent M. leprae, showed heavy growth of the leprosy organism, with definite evidence of pathogenic changes caused by the microorganism. Numerous mycobacteria were found in various tissues of the animal, particularly in the extremities. This exciting finding, if confirmed could be a real breakthrough in the long search for a suitable animal model for study of the pathogenesis of leprosy. It could also be of great value as a readily available source of virulent in vivo-grown leprosy mycobacteria.

Other promising leprosy research efforts deal with study of chemotherapeutic agents and their pharmacological activity in treatment of leprosy patients:

AI-07801-05 - L. Levy
AI-08214-04 - J. H. Peters

Both of these studies are on the most promising of the currently available anti-leprosy chemotherapeutic agent, Dapsone, and its derivatives, monoacetyl dapsone and diamino-diphenyl dapsone. In experimental leprosy and in human patients, the dapsone drugs were found to have relatively long half-lives in vivo, with low rates of urinary excretion. Dapsone may prove to be the drug of choice, both for its effectiveness and its low cost, in the treatment of thousands of leprosy patients in the developing countries of the world. The antibiotic rifampin similarly shows great promise as an effective anti-leprosy agent, but its cost is far greater than Dapsone, therefore limiting its potential use. Drug resistance of M. leprae is also under investigation to determine the potential threat of the development of drug-resistant micro-organisms during treatment of severely disabled lepers.

Tuberculosis

This other major disease caused by mycobacteria has continued to be the focus of a well-developed research program, both from the standpoint of the fundamental aspects of the pathogenesis and host-reaction to the various mycobacterial fractions and from the point of applied studies on chemotherapy.

AI-06578-06 - W. Lester

An evaluation of the efficacy, toxicity, and adverse reactions of intensive chemotherapy regimens in patients treated with isoniazid is the major aim of this research. Newer drugs, such as rifampin and capreomycin, show considerable promise in well-controlled studies in the treatment of patients with multiple resistance to the common anti-tuberculosis drugs. No serious adverse reactions or toxicity to the antibiotics was observed. In view of past experience, however, it can be expected that the drug-resistant tuberculosis strains will most likely develop resistance to these new antibiotics also, so that the long-term outlook for patients infected with multiple drug-resistant mycobacteria is rather bleak.

AI-09482-03 - P. Minden

Using quantitative binding tests with radioiodine labeled sera, binding to components derived from M. tuberculosis was observed by all sera obtained from normal as well as tuberculosis patients. The universal occurrence of these antibodies suggests that most if not all persons have been functionally immunized, to some degree, to the tubercle bacillus. Current data also suggest that some of the binding by normal human sera to mycobacterial components may be a result of nonmycobacterial (taxonomically unrelated) immunogens. The possibility arises that resistance to tuberculous infections may be due in part to immune mechanisms, whether cellular or humoral, that have been stimulated by nonmycobacterial organisms.

Streptococci

Rheumatic heart disease is the most common form of heart disability in children. A resurgence of this disease is being seen clinically in children at the present time.

AI-09645-02 - E. M. Ayoub

The role of the streptococcal Group A carbohydrate and the antigenic cross-reactivity with glycoproteins found in the heart valve were explored. Results of this study showed that there is indeed a strong relationship between antibody to the streptococcal Group A carbohydrate and the valvular tissue. Surgical manipulation of diseased valves in rheumatic patients caused a significant rise in Group A antibody levels. Conversely, complete excision of the diseased valve resulted in a significant decline of the circulating antibody levels.

AI-06487-07 - D. W. Watson

Dr. Watson has previously described a pyrogen-producing exotoxin in strains of Group A streptococci. Although not yet fully characterized, one of the more remarkable properties of this pyrogenic exotoxin appears to be its ability to enhance significantly the susceptibility of experimental animals to Gram negative bacterial endotoxins.

Urinary Tract Infections

AI-10668-01 - W. R. Fair

This unique research program has centered on the observed antibacterial effect of prostatic fluid, in an effort to clarify the role of the normal prostate in the prevention of urinary tract infection. Spermine and prostaglandins, which are normal constituents of prostatic fluid, were excluded, on the basis of this study, as being the active agents. A substance called Prostatic Antibacterial Factor (PAF) was identified in the prostate fluid of normal males. This potent antibacterial material, active against a variety of microorganisms commonly found in urinary tract infections, appears to be a heat stable, non-protein substance of low molecular weight.

Fungal Infections

AI-06931-06 - R. G. Quie

An apparent association of fungal septicemia with prolonged intravenous feeding of hospitalized patients has been found. Research on identification of common contributing factors and the causal organisms showed that the major fungal agent involved is the yeast Candida albicans. The frequency of septicemia was clearly demonstrated to have increased in proportion to the duration of intravenous feeding; these patients showed a definite increase in fungal rather than bacterial septicemia. Results of these studies indicated that greater attempts to improve methods of intravenous fluid handling and administration to patients are needed.

AI-03082-12 - M. G. Koenig

"Pigeon breeders disease", or cryptococcosis, is caused by a fungus usually isolated from old birds' nests or soil heavily contaminated with bird droppings. Inhalation of contaminated airborne dust particles is a common method of spread of this fungal infection. The disease, when untreated, usually develops into a serious meningeal infection. Diagnosis of cryptococcal meningitis is difficult, especially of chronic meningeal infections. A new diagnostic detection test, based on agglutination of latex particles by patients' serum or spinal fluid in the presence of a cryptococcal antigen, has proven to be very successful. In some patients, this technique may be the only means of diagnosing the disease while the patient is still alive. Early detection is important, so that therapy with amphotericin B can be promptly instituted.

K3-AI-13,188-04 - G. S. Bulmer

Results of Dr. Bulmer's study indicate that cryptococcal yeast cells inhaled by guinea pigs via aerosols were killed in the lungs, but not cleared by the lung macrophages. Myeloperoxidase from peripheral leukocytes of either test animals or humans kills the non-encapsulated cells of C. neoformans. Apparently, the lung macrophages lack myeloperoxidase. Another interesting development in this research is the finding that certain C. neoformans strains, carried for many years in laboratory cultures, caused tumor development in mice. Large lesions, called "cryptococcomas", were found in the central nervous system of the injected mice; these tumors may be caused by fungal strains of decreased virulence.

AI-07791-05 - J. P. Utz

Cryptococcal isolates from human meningitis infections were studied in experimental animals. The presence or absence of capsules did not alter the final outcome of these infections; however, mice infected with capsulated strains showed earlier development of lesions. New drug therapy studies indicated that 5-fluorocytosine (5 FC) taken orally has great promise in inhibiting growth of the infectious yeast. Combined therapy with 5 FC and amphotericin B seems to offer the best results for seriously ill patients.

AI-07461-05 - D. H. Howard

Intracellular growth of Histoplasma capsulatum was found to be inhibited within the monocytes of immunized mice. Results of this study show that immunity to histoplasmosis is lymphocyte-mediated; macrophages can be activated to inhibit H. capsulatum by addition of lymphocytes to cell cultures already parasitized by the fungus. The mechanism of the growth inhibition and of macrophage activation is still under investigation.

AI-08815-04 - D. W. Mackenzie

Dr. Mackenzie has found that specific epidemiological types of Candida albicans could be defined by specific markers. In a study of a hospital nursery, it was found that colonization of infants' skin was achieved by more than one epidemiologic type; sometimes two or three different strains could be isolated from different sites on a single child. No single strain characteristic, however, could be isolated which correlated with virulence of the yeast.

C. BIOCHEMISTRY AND PHYSIOLOGY

The approximate level of support given biochemistry and physiology:

Type of Grant	Number	Amount
Research grants	313	$14,477,000
Training grants	7	446,000
Fellowships	37	644,000

Summary

Antiviral substances continues as the sole Special Emphasis Program of this Branch, although interferon is the only component of the program accorded an advantage. As of January 31, 1972, the Institute had an investment of approximately 1.5 million dollars in interferon research. Another component of the program which is not accorded a funding advantage, however, is the area of bacteriophage research in which we had 4.3 million dollars invested as of January 31, 1972.

During the summer of 1971, it was decided to segregate grants in the area of basic animal virus research in the following:

1. Structure and assembly
2. DNA synthesis and recombination
3. RNA synthesis (transcription)
4. Protein synthesis (translation)
5. Membrane phenomena (invasion and release)
6. Cell response (permissiveness and shut-off)

This was done after consulting with and obtaining the views of leading workers in this field.

A grid was prepared for the November 1971 meeting of the Council, and subsequently for the March 1972 meeting, at which time it was ascertained that we had an investment of 1.8 million dollars in this important area of research. No special funding advantage is given to grants in this area at present.

RESEARCH GRANT HIGHLIGHTS

AI-02095-14 - C. Schaffner

Dr. Schaffner has made recent advances in an area of importance to this Institute. The polyene macrolide antifungal antibiotics have been known for two decades as interesting metabolites with a high degree of activity against many yeasts and fungi. Several of them, including candidicin, amphotericin B, nystatin, and trichomycin have become clinically important. The physical characteristics of these antibiotics are such that oral and topical formulations were possible, but applications by the parenteral route were greatly hindered by their inherent great insolubility in water. It was generally observed that these antibiotics were highly unstable to a variety of conditions, including temperature, pH, oxidation, reduction, light, etc.

Dr. Schaffner was able to prepare methyl ester derivatives of certain of these antibiotics, and these derivatives generally exhibited excellent water solubility and good partial retention of antifungal activity. Following this, he prepared ethyl esters of certain of these antibiotics with similar results involving increased water solubility and excellent retention of antifungal activity.

This work represents an important breakthrough in fungal diseases which may lead to an extensive clinical application.

AI-05629-10 - T. Merigan

Dr. Merigan continues his studies on the role of interferon in host resistance in vivo in known viral infections. Increased evidence has been obtained for a role of local interferon in recovery from viral infections, especially in persistent viral infection such as disseminated herpes virus infections. This effect is most clear cut in humans with immunocompromise (Hodgkin's disease, etc.) and cellular immunity is often impaired in these patients. He is turning to a careful study of cell-mediated immunity and interferon interrelationships which is being approached both by in vivo studies following the course of cell-mediated immunity to specific viruses in man, as well as in vitro study of the cells interacting in the production of interferon and transformation of lymphocytes following exposure to specific viral antigens. The same cellular requirement for demonstration of transformation associated interferon which was shown previously in his laboratory in relationship to phytohemagglutinin has been found for the purified protein derivative tuberculin and most recently for heat-inactivated vaccinia antigen with cells from appropriately sensitized humans in their study. Currently, the assays for interferon and other so-called mediator substances production by lymphocytes as triggered by viral antigens are being developed for both herpes simplex and varicella-zoster. It is his belief that only by study of cell-mediated immunity to specific viral infections might one be able to unravel the role of cell-mediated immunity in particular viral infections and the influence of various treatment modalities on that process.

As a model for immunocompromised humans, various infections are being studied in chickens who have either undergone hormonal bursectomy or neonatal surgical thymectomy. The course of infection and various immune responses in these chickens versus their normal counterparts should provide information on the role of various host defenses to particular virus infections. Recent studies with birds have employed either avian influenza or Rous sarcoma virus, and future studies will be concerned with vaccinia as a more appropriate model for human DNA viral cutaneous infections. In addition, studies are being carried out on interferon effects on several tumors, including feline leukemia virus infection and dog lymphoma in collaboration with Dr. Lloyd Old and his associates at Sloan Kettering Institute.

AI-07877-05 - R. Collier

Dr. Collier continues his careful work involving the molecular mechanism of the pathogenicity of diphtheria toxin for which he will be given the Eli Lilly Award at the forthcoming meeting of the American Society of Microbiology in April 1972. The major goal of his research efforts has been a clearer under-

standing of the structure of the toxin in relation to its toxic and enzymatic activities. The toxicity of diphtheria toxin is believed to result from its capacity to inhibit protein synthesis. The inhibition results from inactivation of transferase II, one of the peptide chain elongation factors, by a reaction involving adenosine diphosphate ribosylation of the transferase with nicotinamide adenine dinucleotide as substrate. The toxin acts catalytically in this reaction, and transferase II is the only known protein substrate. A two-step activation process is required in order that the toxin express its enzymic activity.

AI-08078-05 - T. Watanabe

Dr. Watanabe, the pioneer in the discovery of the resistance factor, is continuing his research on the evolutionary relationships of resistance factors. A continued increase of multiple drug-resistance factors in man and animals is causing a serious difficulty in the chemotherapy of bacterial infections involving bacteria carrying R factors. This increase is obviously due to selective pressures exerted by the antibiotics used for man and animals. The use of antibiotics for animals has been a subject of much debate for the past several years and the development of new antibiotics which are effective as feed additives for animals and yet do not promote the increase of R factor-carrying bacteria has been strongly anticipated. Dr. Watanabe has found that flavomycin, an antibiotic, is highly effective against bacteria carrying R factor, and less effective against bacteria without R factor. This discovery is not only of great basic interest, but may have practical application in the development of a new and safer animal additive.

AI-09921-02 - G. Reeke

Dr. Reeke has made considerable progress with the crystallographic structure determination of concanavalin A, a plant lectin which binds polysaccharides and stimulates lymphocyte transformation. Current interest in this substance centers on demonstrated differences in its effects on binding to various kinds of cells, including normal and tumor cells, implying differences in the surfaces of these cells. Understanding of the molecular basis for the effects of concanavalin A on cells mediated by interaction of concanavalin A with cell surface structures is of great interest in cell biology, and is of particular importance in understanding and exploiting functional differences between normal and tumor cell surfaces.

AI-09152-05 - J. Strominger

Dr. Strominger has made considerable progress in understanding the mechanism by which penicillin kills bacterial cells. Perhaps the most noteworthy progress during the year has been the finding that some bacterial cells contain multiple components which react with penicillins. At least one of these conponents in Bacillus subtilis is a D-alanine carboxypeptidase. Evidence has been obtained, however, that inhibition of this enzyme by penicillins is not lethal for the microbial cell. This D-alanine carboxypeptidase has been purified to homogeneity and it has been shown that one of its four sulfhydryl groups disappears on reaction with penicillins.

D. PARASITOLOGY AND MEDICAL ENTOMOLOGY

The approximate level of support given parasitology and medical entomology:

Type of Grant	Number	Amount
Research grants	79	$6,434,000
Training grants	21	1,249,000
Fellowships	16	287,000

Summary

Schistosomiasis, malaria and amebiasis continue as the diseases supported most heavily by the research grant budget of this Branch. A number of investigators are searching for means of controlling snails which are the vectors of schistosomiasis. Others are studying various aspects of immunity to this disease in hopes of eventually being able to develop a vaccine. A number of studies of the metabolism of the schistosome parasites are aimed at finding new pathways for attacking the parasite by therapeutic drugs. In studies of malaria, the greatest emphasis is placed on immunity in hopes of eventually producing a vaccine. In studies of amebiasis, the emphasis is on better diagnosis and new methods of treatment. In both these aspects, considerable progress is being made.

Toxoplasmosis has come to public notice recently. Investigators supported by research grant funds of this Branch were among those who recently brought to light new knowledge of the phases of the life history involving the cat. Studies are continuing to elucidate additional details of the lift history, and emphasis is now being placed on immunological aspects of the infection. It is possible that vaccination of cats can be effective fairly soon, to be followed at some later date by immunizing women who become pregnant or wish to do so. Thus, the danger of congenital infection might be eliminated.

The Special Emphasis Programs of this Branch have been reviewed by ad hoc committees of specialists and the definitions narrowed and restated. These new definitions have been approved by the Advisory Council of the Institute. The Special Emphasis Program "Immunity to Animal Parasites" now covers studies which lead toward possible vaccination against diseases caused by animal parasites. The Special Emphasis Program "Biological Regulation of Vectors" is concerned with basic science projects which have a potential of leading to new methods of biological control of vectors of human disease. In each of these programs, approximately one million dollars is provided from the research grant budget of this Branch.

RESEARCH GRANT HIGHLIGHTS

AI-09363-03 - E. Van Handel

Dr. Van Handel and Dr. Nayar have studied the use by mosquitoes of various chemical compounds as fuel to sustain flight. In general, sugars rather than fats are used. The availability and rates of utilization of various compounds have been related to the ability of mosquitoes to undertake extensive migration.

Facts regarding the mechanisms involved in dispersal of mosquitoes are valuable for epidemiologists studying the spread of vector-borne diseases of humans.

AI-06262-08 - E. J. L. Soulsby

Dr. Soulsby and associates have studied cutaneous leishmaniasis in the guinea pig. The reactions to antigens derived from these parasites indicate that cells are mobilized at the site of infection through migration from bone marrow and elsewhere. The activation of these cells apparently enables them to destroy the parasites more effectively. Further studies along these lines could lead to possible means for vaccinating against this serious and widespread disease.

AI-04919-10 - P. Beaver

Dr. Kalkofen, supported by a research grant of which Dr. Paul Beaver is principal investigator, has studied the effects of the drug Dichlorvos on hookworm larvae developed from eggs passed in stools. This drug has recently come into use for treatment of hookworm disease in humans and dogs. The results of this study indicate that the drug is not only successful in eliminating the adult hookworms from the bowel, but the larvae hatching from eggs passed after treatment do not live. Thus, the treatment has a secondary effect of sterilizing the soil at defecation sites and preventing reinfection or infection of others coming in contact with the contaminated soil.

AI-02631-14 - F. van Lichtenberg

Dr. Lichtenberg and associates have made an exhaustive study of the pathology of urinary schistosomiasis. In addition to voluminous information concerning the pathogenesis of the disease, they have found a direct relation between the intensity of infection, severity of disease, and prevalence of complications. They point to the need for quantitative evaluation of egg output as well as accurate clinical diagnosis in endemic areas of schistosomiasis hematobia.

AI-07744-05 - L. W. Chi

Dr. Chi and associates have shown that hybrids between species of snails transmit schistosomiasis japonica if one parent strain was able to transmit the same strain of parasite. For example, a species of snail from the mainland of China will transmit all strains of the parasite which infect humans. The species from Taiwan is susceptible only to the non-human strains of parasites found in domestic and wild animals in Taiwan. Hybrids between these two species of snails are susceptible to both human and non-human parasites. These results have great significance in the epidemiology of these important diseases, and could have value in the possible biological control of snail vectors.

AI-09654-03 - R. Elsdon-Dew

Drs. Elsdon-Dew and Powell have concluded from controlled clinical trials in South Africa that metronidazole is the drug of choice in treating severe amebic dysentery or amebic liver abscess. They further conclude that it is

a mistake to follow the advice of some clinicians and prescribe a smaller than normal dose of this drug for amebic infections of the intestine which are asymptomatic or which show only mild symptoms. They state that their results indicate that in spite of the apparent cures obtained by those who advocate these smaller doses, the results are unreliable and do not assure eradication of infection from the bowel.

AI-07857-06 - C. W. Schwabe

Dr. Schwabe and colleagues have studied the records of 82 California hospitals and found that there were 69 cases of hydatid disease in a 10-year period. Most of these were among sheep herders of the central valley. Other cases were among immigrants. It is recommended that this disease should be included among the notifiable diseases in California and that greater attention should be given in teaching physicians to the necessity for use of more sensitive tests now available.

E. VIROLOGY AND RICKETTSIOLOGY

The approximate level of support given virology and rickettsiology:

Type of Grant	Number	Amount
Research grants	162	$7,506,000
Training grants	31	1,784,000
Fellowships	33	573,000

Summary

Viral diseases are still among the most serious infections affecting the health of people and undoubtedly present the largest variety of problems to be surmounted before marked improvement is made against infectious diseases today. The two Special Emphasis Programs of the Virology and Rickettsiology Branch, namely the Chronic and Degenerative Diseases of Man and the Viral Hepatitis program, showed some progress this past year with encouragement that further advances may be expected in the not-too-distant future. The work with the Viral Hepatitis program, especially with Australia antigen (Au), is continuing to show progress in rapid detection for improved blood screening programs as well as possible methods of giving some protection to those receiving blood transfusions, which will help eliminate one major source of the disease. Work with the respiratory virus problems continues to receive considerable emphasis with progress being made with trial influenza vaccines, and encouragement for a good immunization program before another pandemic.

RESEARCH GRANT HIGHLIGHTS

Chronic and Degenerative Diseases

The chronic and degenerative diseases of man continue to present very difficult and time-consuming investigations on viruses or virus-like agents causing diseases. Important research is being conducted on natural and experimental animal diseases which, although not a substitute for direct human studies, parallels the chronic and degenerative diseases of man.

AI-09023-04 - D. Walker

A papova-like virus was cultivated from the brain in a case of progressive multifocal leucoencephalopathy (PML) complicating Hodgkin's disease. This newly isolated virus (called JC virus) apparently commonly infects people in Wisconsin as revealed by serologic surveys for antibodies in people in that state. Infection apparently occurs in childhood and 60 percent or more of the adult population have antibody against the virus.

AI-06477-11 - J. B. Henson

Dr. Henson reported results on Aleutian disease in mink which indicate that a virus may persist in a host in which the agent is widespread in the tissues, accompanied by a pronounced antibody response. Whether these antibodies are partially effective in limiting the amount of virus remains to be clarified.

Instead of being hyporesponsive, the infected mink appears to be hyperresponsive, making large amounts of antibody. The significance of the production of such large amounts of antibody remains to be elucidated. These studies further incriminate the host's immune response in the pathogenesis of glomerulonephritis and arteritis.

AI-09484-03 - M. B. Oldstone

In the work on the immunopathology of persistent viral infections, considerable information is added to the concept that pathogenesis of tissue injury in persistent viral infections is caused by the interaction of host-immune response against the virus in virus-infected tissues.

AI-07471-06 - J. B. Henson

Equine infectious anemia (EIA) provides one of the best models for a chronic viral disease with recurring exacerbations. The persistence of EIA virus in infected horses for the life of the animal establishes the chronic nature of the disease. The mechanism of viral persistence in this disease could well be applicable to many other persistent viruses of man and animals. The type of host-defense mechanism involved against equine infectious anemia virus, and the failure of these defense systems to eliminate the virus from infected horses, are unlikely to be restricted to a narrow spectrum of animal species. Anemia is a constant feature of EIA virus infection. The studies of this grant indicate that the virus has an indirect role in causing hemolysis and that the red blood cell destruction is immunologically mediated. This type of mechanism has immediate application to many virus diseases of man and animals in which anemias occur.

Vaccination

Continued research is being carried out to give even better and safer vaccines where vaccines now exist which have been recognized as risky for many people, and are the cause of anxiety despite the protection given.

AI-09706-02 - H. Koprowski

This grant's long-range goal is to investigate the biology of rabies virus and its by-products with the ultimate aim of developing an effective vaccine for man and animals, both for widespread prophylactic immunization and post-exposure treatment. Results of tissue-culture vaccine indicate very good promise for prophylaxis in man with one dose or perhaps a few doses, of highly concentrated, purified tissue-culture vaccine.

AI-09769-03 - P. L. Ogra

Rubella antibody activity in serum, nasopharynx and joint aspirates, and recovery of rubella virus from joint aspirates were studied in three previously well children, and one child with pre-existing rheumatoid arthritis, who developed repeated episodes of arthritis with effusion for several months following parenteral immunization with live attenuated rubella vaccine (HPV-77DK/12). Rubella virus was recovered from the joint aspirates of all

previously well children for as long as three to four months after immunization with rubella virus vaccine. The investigators suggest that the recovery of rubella virus during subsequent recurrences of arthritis may indicate a distinct predilection of the rubella virus for the joint tissues and the continued replication of this virus in the affected joints. Such a selective distribution of virus has been noted following immunization with other vaccines.

Infectious Hepatitis

Infectious hepatitis is a disease of major public health importance, with approximately 70,000 reported cases being detected and over 1,500 deaths each year in the United States. This disease has been the target of intensive research by many capable investigators with only limited recent success. New and fast-breaking developments during the past few years have provided an opportunity for detection and characterization of one of the agents causing serum hepatitis. The discovery of Australia antigen, also called hepatitis-associated antigen (HAA), has provided a means of screening blood to prevent transfusion hepatitis. Advances are being made with more sensitive and rapid methods for detection.

AI-10176-02 - P. F. Kohler

The broad objective of this project is a delineation of the immune responses to the hepatitis-associated antigen (HAA) in normal individuals who have recovered spontaneously as compared to those who develop persistent HAA; i.e., chronic carriers. Extensive study of 25 positive blood donors included complete liver functions, serum complement, skin tests to five antigens evoking cellular immune responses and, in ten selected instances, liver biopsy. In four individuals who were clinically normal by liver function tests and on physical exam, the biopsy revealed changes ranging from hepatocellular pleomorphism to inactive postnecrotic cirrhosis. Thus, even in clinically normal HAA carriers, definite evidence for liver disease is present.

In four women with antigen positive hepatitis at the time of delivery, the cord blood from their infants was negative. Within four to six weeks, each neonate developed HAA, however, which has persisted for six to 12 months. This suggests that the carrier state may be acquired during the early newborn period.

F3-AI-47,968-01 - R. Ward

Post-transfusion hepatitis is now recognized as a serious threat to the convalescence of patients having illnesses which require transfusion. It has been estimated that of approximately two million persons a year receiving transfusions, 30,000 develop hepatitis, and 1,500 to 3,000 die of the disease. The investigators gathered substantial evidence that transfusion of blood preincubated with gamma globulin modified for intravenous administration reduces the incidence and severity of hepatitis. Suggestions are made as to possibilities for meeting the demands on the current gamma globulin facilities with this gamma globulin usage.

Common Cold

The common cold (Viral Respiratory Infections) continues to be the largest single acute disease problem of the general population. Research over a considerable period of time has produced much information regarding the causes of these infections, but much more is needed before practical control can be anticipated. However, new techniques that give a better understanding of the natural mechanisms of resistance and immunity are being developed that give encouragement to finding better approaches for prevention and cure of these viral infections.

AI-09775-03 - J. P. Fox

Significant data regarding rhinovirus (infections in families) are emerging from studies of these viruses. One of the most striking observations is the large number of serotypes circulating in the community at a given time. For example, 48 strains isolated with a period of one month represented 15 serotypes. A high rate of subclinical infections was recorded. Significant progress has also been made in demonstrating further antigenic relationships among the existing rhinovirus serotypes, and investigation of antibody response in rabbits to purified virions and fractions or rhinovirus preparations. This type of accumulated information is a necessary basis for an approach to control of these infections.

Influenza

There has been renewed interest in influenza research following the recent epidemics of Asian influenza, and the knowledge that another pandemic may take place in a few years. These have been recorded as occurring at periodic intervals. Continued influenza investigations remain in order to better understand the underlying causes of these epidemics and the resulting high morbidity and mortality that usually occur from these viruses and/or interactions with bacterial agents.

AI-09304-04 - E. Kilbourne

The X-31 recombinant influenza virus vaccine developed under this grant is now in commercial production in the United States, as well as in a number of foreign countries. It will be used in the military formula vaccine in the present year. It is worth noting that this is the first hybrid, or recombinant virus used for immunization of man against any disease. A new recombinant vaccine, X-32, is in pilot production and is being used to immunize volunteers. This vaccine embodies a new approach to immunization for influenza. The presence in the recombinant virus of only one minor antigen, the neuraminidase, which is relevant to immunization against the Hong Kong virus, provides "mono-antigenic immunization" which is expected to provide limited and partial protection against the disease. On the basis of animal studies, it is predicted that such immunization will not prevent infection with influenza virus, but will prevent the evolution of infection to disease. Thus, the subject so immunized may realize the benefits of immunity engendered by natural infection as well as the partial immunity induced by this method of vaccination. This is an example of research developing new vaccine materials that will increase their effectiveness.

AI-05600-10 - P. Choppin

Paramyxoviruses are the etiological agents of several acute diseases of man. There is increasing evidence of their importance in chronic diseases, which is well exemplified by the role of measles virus in subacute sclerosing panencephalitis. Influenza virus remains as the most important disease of man in terms of overall morbidity and mortality. This project is gaining and accumulating much information about the structure, replication, and pathogenesis that is so essential for effective control of diseases produced by these viruses. The central role of cell membrane changes in a variety of human diseases, including chronic neurological diseases and cancer, emphasizes the great need for a better knowledge of membrane structure and biogenesis. The enveloped viruses investigated here provide good models for study and these studies have already contributed useful information to the general field of membrane biology.

Arboviruses

The arboviruses continue to maintain an epidemic threat to large segments of our populations, and diligent surveillance and research are needed to keep abreast of problems arising with these viruses. Support of investigations on these viruses are needed to help clarify a number of the basic problems. This should lead to better control procedures where large human populations are annually susceptible to infections and possible epidemics.

AI-03028-13 - W. C. Reeves

Previous studies on this grant had indicated the disappearance of Western equine encephalitis (WEE) virus from Kern County, California, in 1969 which coincided with the sudden emergence of a population of Culex tarsalis mosquito that was resistant to licensed organophosphorous insecticides. The hypothesis was then advanced that C. tarsalis resistant to organophosphorous insecticides were inefficient vectors of the WEE virus. This hypothesis was evaluated by virus feeding experiments in the laboratory and all mosquitoes of this specie were extremely resistant to infection with WEE virus, including C. tarsalis from a colony that had been maintained in the Bakersfield laboraotry for years. The colony had not purposely been exposed to insecticides, but undoubtedly had had some exposure to organophosphorous insecticide in the environment where it is located. Also, it was observed that C. tarsalis that were highly resistant to organophosphorous insecticide were highly autogenous (i.e., laid eggs without taking a blood meal). These studies are being pursued further. The persistence of this type resistance to infection by this major vector of Western encephalitis virus in that part of California would materially reduce the risk of this type human infection occurring.

AI-00771-18 - R. Hanson

Among the notable contributions of the work supported by this grant have been the demonstration that the geographic range of Eastern Equine Encephalitis (EEE) extends into the center of the North American continent; that LaCrosse virus, a member of the California encephalitis (CE) group, currently probably the most important epidemic arthropodborne disease in the country, is a significant cause of encephalitis in children; that the important vector of LaCrosse

virus is the Aedes triseriatus mosquito, and the probable wildlife host is the eastern chipmunk; and that three other California group viruses are present in Wisconsin and each appears to have its own vector and wildlife host.

Other Viruses

Findings obtained from studies of a number of the other virus diseases give quite interesting results on methods of replication, isolation, and characterization of the causal agents of a number of infections, etc., that are important in the accumulation of information for a better understanding of such viral infections and the anomalies caused by them.

AI-02316-14 - S. E. Sulkin

Investigations on this program support the hypothesis that certain characteristics of rabies virus infections, such as the often prolonged incubation periods in man and other animals, the persistent, inapparent infections in bats and other natural hosts, and the endosymbiotic infections which can be established in certain tissue culture systems, indicate that information accumulated through studies of rabies virus host cell interactions in vivo and in vitro may be applicable to a better understanding of latent and inapparent virus infections in general. These may also apply to the intriguing "slow virus" interactions that are so difficult for researchers.

AI-07746-05 - E. B. Shotts, Jr.

Using a "model" system developed for the study of interrelationships of swine influenza virus and the helminth, Strongyloides ratti with mice and rats, the investigator was able to demonstrate the transmission of virus by S. ratti from mouse to mouse or rat to rat, but it is not yet possible to state at this time whether the worm acts as a mechanical or biological vector. The swine influenza virus has been demonstrated by flourescent antibody (FA) in the first and second state feeding larva exposed in vitro or exposed in vivo, which is the first direct evidence that virus enters the worm in this sytem.

K4-AI-46,206-03 - R. D. Feigin

During the course of studies to assess the effect of Coxsackie B3 virus on the blood of amino acids of Schwenkter strain mice, the investigator noted an apparent difference in the susceptibility of mice to this virus which depended on the time of day that the animals were challenged. In separate experiments, he then inoculated groups of Charles River mice with Coxsackie B3 virus every four hours around the clock, beginning at 0800 hours, with adequate controls also being maintained. From these experiments, it was apparent that challenge of the Charles River mice, of equivalent weight housed under identical conditions, was dependent upon the time of day that challenge is effected with the virus. Infection in these experiments appeared to be more severe following the challenge at 1600 hours than following the challenge at 0400 hours.

III. Research Grants Program Analysis

Fields	1972 Estimate Amount
Allergy and Immunology	$14,546,000
Lymphocyte Biology	(635,000)
Mechanisms of Immunity to Infectious Agents	(419,000)
Cell-Mediated versus Humoral Antiviral Responses	(820,000)
Bacteriology and Mycology	17,695,000
Mechanisms of Drug Resistance	(1,139,000)
Streptococcal Disease and Sequelae	(1,574,000)
Basic Biology of Venereal Disease	(456,000)
Parasitology and Medical Entomology	7,218,000
Immunity to Animal Parasites	(1,539,000)
Biological Regulation of Vectors	(1,236,000)
Virology and Rickettsiology	14,386,000
Viral Hepatitis	(573,000)
Chronic and Degenerative Diseases of Man	(905,000)
Interferon	(1,526,000)
Subtotal	$53,845,000
General Research Support	$ 5,149,000
Scientific Evaluation	42,000
Categorical Clinical Research Centers	1,252,000
International Centers for Medical Research and Training	2,399,000
Subtotal	$ 8,842,000
Total, Research Grants	$62,687,000

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COLLABORATIVE RESEARCH PROGRAMS: SUMMARY

NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES

FY 1972

During FY 1972 there were a number of organizational and personnel changes of significance to Collaborative Research. As a result of decentralization of NIH contracting activities, the NIAID Contract Management Branch was given the responsibility for negotiation and award of Institute contracts. Mr. Seering and Mr. Harris of the Collaborative Research administrative staff were transferred to the Contract Management Branch where their new activities include preparation and processing of program documents and negotiation of contracts for the Infectious Disease Branch and the Transplantation and Immunology Branch.

Dr. June Dunnick joined the Collaborative staff in September 1971 and was assigned to the Infectious Disease Branch where she has been instrumental in developing and managing contracts on hepatitis. Also in September Dr. Daniel Mullally entered an eight month intensive training program in epidemiology and biostatistics at the Johns Hopkins School of Public Health and Hygiene. During his absence Dr. George Galasso has served as Acting Chief, Infectious Disease Branch.

Mr. Robert Pennington left the Research Resources Branch in April 1972 to accept a position in the Blood Resources Program of the National Heart and Lung Institute. Dr. Robert Scott replaced Dr. Douglas Brand as Medical Officer in the Transplantation and Immunology Branch on July 1, 1971.

Mr. John Hannan, Administrative Officer for Collaborative Research is presently detailed to the Financial Management Branch where he is assisting in the formulation of new travel policy and procedures. Mrs. Doris Parkinson, Administrative Officer for the SEATO Cholera Research Program is responsible for the Collaborative Research administration.

Despite shortages in personnel, it was possible to support some training. In addition to Dr. Mullally's training at Johns Hopkins, several of the staff received training in administration, contracting and computer application.

The three formal advisory committees for the Collaborative Research Program have functioned well in their broadened role of evaluating both ongoing and proposed Institute programs. Increasing utilization of subcommittees and consultants has been effective in obtaining advice on more specific scientific and technical issues. A Subcommittee on Influenza was established late in FY 1971. The Arboviruses Committee was terminated at the end of FY 1971 and its members were amalgamated into the Research Resources Committee.

The Transplantation and Immunology Branch is placing increased emphasis and effort on evaluating the effectiveness of tissue matching between donors and recipients of organ transplants as a means of predicting organ survival by providing preloaded trays of high quality reagents and an analysis of

training results. Another area receiving increased program support is the production and animal assay of anti human thymocyte serum, with the expectation of future evaluation as to its effectiveness in increasing survival of transplanted organs in man. It is anticipated that these two major evaluation programs will take 2-5 years to complete.

Sponsorship of a collaborative study of soluble antigens of human leucocytes (HL-A) and mice (H-2) is being phased out during the coming year.

The Transplantation and Immunology Branch continues to sponsor studies on freeze drying of reagents, immunosuppressive drugs and research on the immunologic responsiveness of recipients of transplants. Collecting and distribution of scientific information are designed to complement the principal program activities.

The Research Resources Branch has expanded its scope to include a variety of resource-type programs such as the NIAID/AEC sponsored Molecular Anatomy Laboratory, the production, distribution and evaluation of ragweed antigens; and the Smithsonian Institute mammal identification services. The production of conventional seed and antisera to most of the viruses and mycoplasma of human health research is now essentially complete. Program activities will now be focused on producing more sophisticated types of reagent that until recently were not feasible. These newer reagents include antisera to influenza virus neuraminadase and hemagglutinins and, in the case of hepatitis, antisera for hepatitis antigen subtypes and other antigens and particles associated with hepatitis B.

During FY 1972 the Infectious Disease Branch assumed responsibility for developing and implementing a long-range collaborative program on hepatitis. Through a series of workshops and planning sessions, the Branch has developed a program for initiation of projects on epidemiology, a hepatitis reference laboratory, and a vaccine development and testing effort which will include integrated studies on animal models, tissue culture, biophysical and biochemical characterization of antigens and agents and ultimately, evaluation of vaccine in man.

The Antiviral Substance Program has progressed to the point of clinical evaluation of promising candidate drugs. Initially herpes-virus infections will be subject to intensive studies with exogenous interferon, interferon inducers and candidate antivirals, iododeoxyuridine and adenosine arabinoside.

The Bacterial Vaccine program includes projects on pneumococcal pneumonia, meningococcal meningitis, H. influenzae type B infections and streptococcal infections. Of these, the pneumococcal program has progressed furthest toward clinical application. Phase III testing of hexavalent pneumococcal polysaccharide vaccine is anticipated for the fall of 1972 followed by a concerted effort to evaluate the total program over the next two years.

Of the viral vaccines under study, greatest emphasis is now on influenza, parainfluenza and respiratory syncytial viruses. Greatest promise appears to be in virus vaccines attenuated for man by selection of temperature-sensitive mutants. Progress towards a mycoplasma vaccine has been enhanced by overcoming a number of complex production and purification problems.

INFECTIOUS DISEASE BRANCH

Summary

The major activities during Fiscal Year 1972 involved a reorganization of the vaccine evaluation centers, reducing surveillance studies and increasing the populations for vaccine testing. The Hepatitis Program was established as a branch activity with a series of workshops and initiation of planning and soliciting contracts for work in epidemiology, clinical studies involving hyperimmune gamma globulin, and initiation of a reference laboratory; support of work on the animal model was increased.

In the respiratory virus vaccine development program, a temperature sensitive (ts) mutant of respiratory syncytial virus is currently being evaluated in children and infants to determine safety, lack of transmissibility, significance of revertants and antigenicity. Live parainfluenza virus types 1 and 3 are being clinically evaluated in children.

The Institute, with advice from the Influenza Subcommittee of the Infectious Disease Advisory Committee (IDAC), is sponsoring a series of workshops, addressing themselves to possible recommendations for the control of a possible pandemic in the late 1970's. An inactivated recombinant influenza virus vaccine (surface hemagglutinin of influenza A/Equine 1 and neuraminidase of the X-31 strain of the Hong Kong variant) and a live attenuated influenza A virus (H_3N_2) vaccine have become available, and it appears that both are capable of inducing appreciable levels of antibody with no untoward effects.

Although the efforts on live adenovirus vaccines are gradually being reduced, types 1, 2, 5, 14 and 21 have been clinically evaluated in adults, and vaccination results in the development of serum antibody. Crystalline subunit adenovirus vaccine (type 5 hexon) is currently being evaluated in adult volunteers.

It has been determined that there is no preponderance of a limited number of rhinovirus serotypes responsible for respiratory disease in the civilian population. This accentuates the difficulty in obtaining a successful vaccine in this area.

Following the licensing of rubella vaccines, the Rubella Vaccine Program was phased out. One contractor continues to monitor vaccine efficacy, persistence of antibody and long-term safety of rubella vaccines, and also the protection afforded by high-titered rubella gamma globulin.

The smallpox vaccine study, designed to determine if a better smallpox vaccine or vaccination procedure could be devised, is accumulating data on four different smallpox vaccines. Contractors follow a common protocol.

Bacterial polysaccharide vaccines continue to be evaluated. Meningococcal group A and C polysaccharide vaccines have been tested in several hundred children. Studies on these vaccines and the H. influenza type b polyribose

phosphate (TRP) vaccine have been expanded for wider clinical evaluation. Central reference laboratories and more sensitive assays have been established.

A single contract for the clinical evaluation of M-protein streptococcal vaccine gathered more data regarding the safety and antigenicity of this vaccine preparation. An efficacy trial of the vaccine in adult volunteers is under way.

Large-scale production of an inactivated Mycoplasma pneumoniae vaccine is currently under way. This vaccine will be tested in adult volunteers challenged with a field strain of the agent to determine protective efficacy in preparation for a field trial.

Thirteen monovalent pneumococcal polysaccharides were produced and six evaluated clinically in approximately 400 volunteers. Phase I clinical studies are in progress with the first hexavalent vaccine. Phase II testing is planned for the fall of 1972. A feasibility study has been initiated with the Kaiser Permanente Medical Group in San Francisco to determine the feasibility of a field test in the summer of 1973. A contract was initiated with the University of California to determine the antibody response in children in preparation for possible studies on the role of the vaccine in preventing meningitis and otitis media in young children.

The Antiviral Substances Program (ASP) continues to serve as a focus for the NIAID efforts in the search for effective antivirals. The Program has been effective in establishing research reagent standards for the interferon research community; new reliable and accurate assay systems for interferon quantitation have been established; and studies are being continued in the areas of interferon production and purification and development of more effective interferon inducers.

During FY 72 there was a shift in emphasis by the ASP to more clinical studies. The value of polyinosinic·polycytidylic acid (poly I·poly C) as a treatment for herpes and adenokeratoconjunctivitis is continuing to be evaluated in a double-blind comparative study with iododeoxyuridine (IDU) and placebo; it is thought that sufficient data will be available with an additional year of study to determine its merit as a treatment for these infections. A study of the role of exogenous interferon in treatment of disseminated herpes zoster is being initiated. It is expected that this study will yield both a possible therapeutic effect and also pharmacological data on interferon treatment. Preliminary data indicate that a systematic study on the therapeutic role of adenine arabinoside (Ara-A) and iododeoxyuridine against herpes encephalitis and Ara-A against disseminated herpes zoster should be undertaken at this time, and the ASP is currently pursuing this possibility.

The major changes for the Infectious Disease Branch (IDB) during Fiscal Year 1972 involved the reorganization of the vaccine evaluation centers, a reduction of effort on live adenovirus vaccines and more extensive efforts on clinical studies of polysaccharide vaccines. The Antiviral Substances Program (ASP) is placing greater emphasis on clinical trials with promising antivirals. During FY 72 the major responsibility for the Hepatitis Program of Collaborative Research was transferred to the Infectious Disease Branch. The Research Resources Branch still maintains responsibility for hepatitis reagents and the Molecular Anatomy Laboratory, and all other aspects of the Collaborative Program are now the responsibility of the IDB.

VACCINE EVALUATION CENTERS AND VACCINE DEVELOPMENT

In the fall of 1971, staff readvertised for clinical evaluation centers, both to reorganize this portion of the IDB activities and also because of the gradual change in emphasis of need from epidemiological surveillance to vaccine evaluation, especially in the pediatric age group. Viral vaccine development was also readvertised because of the need for attenuated respiratory syncytial and parainfluenza viruses with well-defined properties for possible use as vaccines. This problem is being pursued from a viral genetic standpoint.

VIRAL VACCINE PROGRAM

Respiratory Syncytial Virus - Parainfluenza: Respiratory syncytial and parainfluenza viruses are the principal causes of severe lower respiratory tract disease among infants and children. The need for effective vaccines to reduce the morbidity and economic toll of these viruses in infants remains apparent. A temperature-sensitive (ts) mutant of RS virus, developed by the Laboratory of Infectious Diseases, NIAID, is now being evaluated in children and infants.

Twenty-two of 26 subjects immunized with ts mutants (9 months to 6 years of age) excreted virus and all 26 had evidence of infection as shown by virus excretions and/or three-fold or greater rise in nasal antibody. All except three of these children had pre-existing antibody. Infection in infants without prior RSV experience resulted in mild afebrile coryza with no [illegible] signs. Revertant virus was found in three subjects but was not [illegible] by any clearly associated illness. Therefore, this viral vaccine [illegible] being given to additional young children in order to obtain more data on [illegible] lack of transmissibility, frequency and significance of revertants, and antigenicity.

An adapted parainfluenza virus type 1 grown in eggs at the 30th passage level is being clinically evaluated for infectivity in young children. It was about 30% infective in children 6 - 10 years of age as measured by rise in antibody. Two to six year olds have recently been administered the vaccine with no untoward effects. Serological and viral shedding data are not yet available. A concentrated, purified inactivated parainfluenza virus, type 3, vaccine was prepared. More developmental work is necessary in order to stabilize the antigen.

Influenza Viruses: An inactivated recombinant influenza virus vaccine was made which contained the surface hemagglutinin of Influenza A/Equine 1 and neuraminidase of the X-31 strain of the Hong Kong variant. A single parenteral injection of the vaccine produced very high titers of anti-neuraminidase antibody in the serum of volunteers who prior to immunization were seronegative. These volunteers are being challenged with live influenza virus in order to determine protective effect of anti-neuraminidase antibody.

Live attenuated influenza A virus (H_3N_2) vaccines have been developed and tested in adults for antigenicity and safety by the Laboratory of Infectious Diseases, NIAID. TS-1 [E] infected 16 out of 17 volunteers but did not cause influenza illness in any. It is now being evaluated under contract for acceptability, attenuation, potential for spread, antigenicity and ability to protect against subsequent natural challenge in adults and children. These will include phase 1 and phase 2 studies.

The Influenza Subcommittee of the IDAC held 3 workshops during FY 72 dealing with antigenic structure, immunologic methodology and vaccines and immunology. The Subcommittee is addressing itself to the possible pandemic of the late 1970's with a view towards recommendations for research activities leading to aborting or lessening the effects of future pandemics.

Rubella: Investigations of the epidemiology, clinical characteristics and viral immunology of rubella, congenital rubella and rubella vaccines are continuing under one longitudinal study. Although safety of vaccines for individual recipients and their contacts is now reasonably established, potential hazards to the fetus by inadvertent vaccination of the mother during pregnancy and efficacy of rubella vaccine-induced immunity are crucial areas requiring continued study.

Smallpox: The NIAID initiated a collaborative study to try to determine a smallpox vaccine and/or vaccination procedure that would result in reduced morbidity as compared to the current procedure.

Four different vaccines were administered by the percutaneous route at the prescribed concentration of the licensed product and one and two 10-fold dilutions less. Antibody response as measured by the hemagglutination assay was similar for three of the vaccines at all three concentrations. The fourth vaccine did not appear to be as antigenic as the other three. Standardized follow-up studies including revaccination are in progress. Statistical analyses of all of the data generated by this study have been and will continue to be performed by Dr. David Alling, Direct Research Programs, NIAID.

The four vaccines are also being administered subcutaneously at three different concentrations. The vaccines appear to be antigenic. Some of the recipients have had swollen arms and/or erythema at the site of injection. The study code has not yet been broken; therefore, the significance of these reactions is not clear.

While these studies are not expected to provide any data on the more serious complications, such as post-vaccinial encephalitis, they should provide data

which might enable selection of an improved vaccination procedure. A method which carries less risk of some of the complications encountered with present vaccines and methods of vaccination would be particularly applicable to those countries now free of smallpox.

Adenoviruses: Live oral adenovirus type 1, 2, 5, 14 and 21 vaccines have been clinically evaluated in adult volunteers. Each of these five vaccines induces a silent infection of the gastrointestinal tract with the development of serum antibody. Adenovirus type 1, 2 and 5 vaccines may be evaluated in children, the target population for these "pediatric" adenovirus types, if a pediatric formulation can be obtained. Adenovirus type 3 vaccine is now available for evaluation in adults.

A crystalline adenovirus type 5 hexon was prepared through a collaborative effort of Dr. Julius Kasel, Laboratory of Clinical Investigation, NIAID, and Dr. Pereira, Mill Hill, England. The crystalline vaccine is now being evaluated in adult volunteers. If the crystallized hexon is immunogenic, then the final goal of a truly purified adenovirus vaccine will have been achieved.

Rhinoviruses: Eighty-nine distinct serological types and one subtype of rhinoviruses were established by a collaborative effort of contractors participating in the rhinovirus typing program. Eleven candidate rhinovirus prototypes were shown to possess cross-reactions with established prototypes. These cross-reactions were studied further. In the cases where the viruses were shown to be identical, the first isolated virus became the numbered type. Viruses with low-grade to moderate reciprocal relationships were also identified. Whether they will be designated as subtypes or as new types has not been determined.

With the exception of military bases and possibly certain other small defined populations, there is no preponderance of a limited number of rhinovirus serotypes in association with respiratory disease, so a vaccine is not feasible.

Therefore, with the completion of phase 3 of the rhinovirus typing, this program will be phased out.

BACTERIAL VACCINE PROGRAM

Bacterial Meningitis Vaccines: Hemophilus influenzae type b and Neisseria meningitidis, groups A, B and C have been defined as the major causative agents of bacterial meningitis, especially in very young children (under the age of 2 years). There is no evidence that these acute infectious diseases of the central nervous system are diminishing in magnitude. Mortality and the permanent neurological defects in children recovering from the acute episodes of meningitis clearly make this a serious public health problem.

Annually in the United States at least 25,000 cases of meningitis are due to Hemophilus influenzae and 10,000 cases or more are caused by meningococci. Since 90% of the meningitis cases occur in very young children, it is apparent that this is the segment of the population at risk and which would benefit by satisfactory immunoprophylactic regimens. Modern methods in immunochemistry have led to the purification and characterization of immunogens

(polysaccharides derived from the capsular component of the organism). Carefully executed clinical studies in adult volunteers have demonstrated that these vaccines provoke specific antibody that may be protective against infections caused by H. influenzae and group A and group C meningococci.

Under the sponsorship of the Infectious Disease Branch, a central reference laboratory has been established by the contract mechanism. This laboratory has developed a standardized radioimmunoassay procedure, capable of measuring nanogram quantities of antibody protein in serum of children vaccinated with varying doses of H. influenzae type b polyribose phosphate (PRP) vaccine. Antibody levels determined by this sensitive quantitative procedure are currently being compared and correlated with established serological techniques (bactericidal and passive hemagglutination tests) which are less sensitive but have furnished good estimates of the antibody protective activity in animal models. In addition, studies are in progress to determine the H. influenzae type b antibody profile in newborn infants and to compare these levels with infants who have had acute episodes of meningitis, and normal infants who have been immunized with the PRP vaccine. By this indirect approach, it may be possible to approximate what level of antibody can be considered protective.

During the last quarter of FY 72, four contracts were awarded to initiate and expand clinical evaluation of PRP vaccine in approximately 600 young children and infants under one year of age. The object of this phase of the program is to establish safety and antigenicity of the vaccine and to determine optimal immunization schedules with respect to duration of antibody levels after primary immunization and to determine if a booster or secondary immunization will be required. If a satisfactory immune response can be achieved in the infant, serious consideration can be directed to structuring field trials with the vaccine to demonstrate efficacy.

A similar program has been designed and implemented for the clinical evaluation of group A and group C meningococcal vaccines in young children and infants. During the past year the Infectious Disease Branch has sponsored the development of a radioactive antigen-binding test that accurately quantitates group specific serum antibody to group A and group C polysaccharides of Neisseria meningitidis. This procedure is a modification of the Farr technique and uses a double label technique to simplify manipulation as well as to adapt its utilization of a mass screening operation. Preliminary studies have shown that while infants respond in a consistent fashion to vaccination with group A and group C polysaccharides, their antibody levels are of a lower magnitude than observed in adults. For determining antibody levels to group A polysaccharide, the recently developed radioactive antigen-binding test is sensitive enough to detect serum concentrations ranging as low as 0.1 ug/ml.

In a pilot study involving 48 children between the ages of 7 months to 9 1/2 years, there were no significant local or systemic side effects to the administration of either group A or group C by the subcutaneous route. The geometric mean antibody concentrations (ug antibody protein/ml) as determined by radioactive antigen-binding assays were 10.3 ug for the group receiving the A polysaccharide and 14.5 ug for the group receiving the C polysaccharide vaccine. Detectable levels of specific antibody were found in the sera of children 10 to 15 months after vaccination with group A or C antigen.

To expedite the clinical evaluation of meningococcal vaccines in children, three contracts were awarded during the latter half of FY 72 to develop immunization schedules for both group A and group C antigens. Route of vaccination, priming dose, time interval between primary and secondary immunization are the main variables to be explored. Microbial monitoring of the nasopharyngeal carriage to establish the carrier status of the volunteers has been incorporated into the clinical protocols of the several contractors and will provide significant epidemiologic information on frequency and geographic distribution of groupable Neisseria meningitidis.

Streptococcal M-Protein Vaccine: The Collaborative Research Programs of NIAID sponsored a two-day Workshop on Streptococcal M-Protein on November 4-5, 1971. Investigators actively engaged in characterizing purified M-protein antigens discussed new technology in growth of the organism and the application of immunochemical techniques for the qualitative analysis of antigenic determinants that have been isolated from cell wall fractions or preparations made from the whole organism. In addition, criteria were established for evaluating biological potency in animal models, such as the mouse and rabbit.

Studies sponsored at the La Rabida Children's Hospital and Research Center, University of Chicago, have generated more information on the response of children between the ages of 5-13 years to a divalent vaccine of types 1 and 3 M-protein. Immunization was accomplished by the intranasal route. Antibody response was measured by passive hemagglutination, the indirect bactericidal test and a microcomplement fixation assay. Approximately half the children responded to the divalent vaccine with the production of detectable serum antibodies; no anti-M antibodies were detected in saliva or nasal washings of the test group of children. During the coming year, a 12-month follow-up of the serum antibody levels will be made in those subjects that had immediate detectable antibody after primary immunization. The duration of the antibody response must be assessed before an optimal schedule for immunization can be developed.

In a pilot study with adult volunteers, an effort was made to determine protective efficacy of type I-M protein vaccine by challenging with live type 1 streptococci. Preliminary data suggest that the alum-precipitated M-protein vaccine, when administered parenterally, afforded some degree of protection against infectious challenge of homologous streptococci. This immunity-challenge study will be repeated with additional volunteers in order that a definitive conclusion can be reached on protective efficacy of the vaccine.

It is anticipated that efforts will be made to expand this program to include additional serotypes of streptococcal M-proteins as immunogens. The ultimate aim of the vaccine program would be a polyvalent vaccine comprised of M-protein antigens most frequently responsible for the upper respiratory streptococcal infection of children.

Mycoplasma Pneumoniae Vaccine: The large-scale production of an inactivated Mycoplasma pneumoniae vaccine for the Infectious Disease Branch has been arranged through a contract with the Ohio State University. Approximately 10 liters of the vaccine will be prepared by July 1972. Through a contract with Baylor University, adult volunteers are scheduled to be immunized with

the candidate vaccine during August and September 1972. These subjects will be challenged with a field strain of Mycoplasma pneumoniae to determine protective efficacy. If this study proves successful, efforts will be made to clinically evaluate the prototype vaccine in children for safety, reactivity and antigenicity during the latter half of FY 73. With the large supply of vaccine anticipated, preparations are under way for a large field trial in military personnel in the summer of 1973.

Pneumococcal Program: In September 1971 the Infectious Disease Advisory Committee reviewed this entire program and made specific recommendations concerning epidemiological surveillance and selection of certain high-risk groups which offered the best possibility for field testing a polyvalent vaccine for efficacy.

The contract with Eli Lilly and Company for the production of 14 serotypes of pneumococcal polysaccharides has continued to progress satisfactorily during the past year. At least 25 gms of polysaccharide material of types 1-9, 12, 14, 18 and 19 have been bulk-produced and packaged in final containers for human administration. Clinical evaluations of monovalent vaccines 1, 3, 4, 7, 8 and 12 have been performed in approximately 400 adult volunteers. Phase I studies are in progress with the first hexavalent vaccine (types 1, 3, 4, 7, 8 and 12). Phase II and a small field trial are scheduled during the fall of 1972 in a large chronic disease hospital. In collaboration with the Indian Health Service, a similar plan has been developed to field test a polyvalent vaccine in paired communities of Navajo Indians living on the reservation in Arizona.

A feasibility study has been initiated with the Kaiser Permanente Medical Group in San Francisco to determine if a field trial with a polyvalent vaccine can be conducted in subjects 45 years or older. This project includes a stepped-up epidemiological surveillance program. Preliminary evidence suggests that there is enough pneumococcal disease to justify a field test in the summer of 1973.

Through a contract with the University of California, studies are in progress to determine the antibody response of children to monovalent antigens and selected polyvalent vaccines containing polysaccharides most commonly associated with pneumococcal serotypes causing meningitis and acute otitis media in young children.

Assessment of the immune response will be accomplished by performing serology with the hemagglutination test and the radioimmunoassay procedure.

HEPATITIS

The NIAID Collaborative Hepatitis Research Program has as its long-range goal research activities leading to control of both hepatitis A and hepatitis B infection; major emphasis is now being placed on a study of hepatitis B infection, since recent knowledge of hepatitis B antigen and antibody characteristics has made it feasible to define this disease. At this time there is no definitive knowledge of hepatitis A antigen and antibody characteristics. The following is a description of the IDB Hepatitis Program, though the total

NIAID Hepatitis Program includes activities within the Research Resources Branch and Intramural Research.

NIAID Hepatitis Workshops: The NIAID has sponsored a series of workshops on important topics in hepatitis research. The purpose of these workshops has been to gather together a group of experts in a particular field to discuss a specified project and offer suggestions and guidelines for the NIAID Hepatitis Program. Recent workshops in this series included: Hepatitis B Antigen Subtypes, Animal Models, Immune Complexes and Hepatitis, and Immunization.

Animal Models: The development of animal model systems offers an opportunity for characterizing the agent of hepatitis B. The general susceptibility of primates to hepatitis B, as recognized by development of antibody to HB Ag, appears to parallel the phylogenetic scale, with those primates closest to man being the most susceptible. Success in transmitting hepatitis B to primates has now been demonstrated in the chimpanzee and the rhesus monkey. The IDB is continuing to sponsor hepatitis research in animal models, particularly support of studies in the rhesus monkey. As funds become available, studies will be expended to a study of hepatitis A in marmosets.

Reference Laboratory: One of the problems in hepatitis B studies is that it is difficult to get accurate and consistent test results for HB Ag and HB Ab. The NIAID plans to establish a Reference Laboratory whose function will be to test for hepatitis antigens and antibodies by the most sensitive techniques available. This laboratory will also work on developing new and better test procedures. This laboratory will be established as a reference laboratory for all NIH Research Laboratories working in the field of hepatitis and will also serve as a reference laboratory for specific NIAID projects, such as NIAID-CDC (Center for Disease Control) collaborative epidemiology studies.

Epidemiology: The NIAID and CDC are collaborating on an epidemiological survey of hepatitis B in hemodialysis units. The objective of these studies is to determine to what degree hemodialysis patients and staff, and family contacts of these patients, can be classified as "high risk populations." These studies include identification of infecting source, method and prevalence of spread, identification of hepatitis B subtypes, and incubation time of the disease. Laboratory support for these studies will come from the NIAID Hepatitis Reference Laboratory.

Passive Immunization: The NIAID, in collaboration with the Veteran's Administration Hospitals, is studying the effects of high-titered HB Ab gamma globulin in preventing hepatitis B infection in persons experiencing accidental parenteral exposure to this disease. Normal gamma globulin preparations confer some immunity to hepatitis A, but these preparations have little or no effect in preventing hepatitis B infection. Preliminary evidence suggests that gamma globulin containing HB Ab might be effective in preventing hepatitis B.

ANTIVIRAL SUBSTANCES PROGRAM

There are many viral diseases for which there is no prophylaxis or therapy presently available and which constitute serious public health problems. There is, therefore, a need for the development of antiviral substances for

the treatment of these diseases. Planning for a program to help meet this need began in July 1969. A thorough study of the field revealed that there was considerable increasing interest in this area and that interferon or interferon inducers were the most promising antivirals to exploit. This continues to be the case, although there have been a number of case histories published recently which indicate that adenine arabinoside (Ara-A) and iododeoxyuridine (IDU) have been effective against herpes encephalitis. The Antiviral Substances Program has therefore held several meetings this current fiscal year to assess the state of the art in preparation for initiating clinical trials to properly determine the efficacy of these drugs.

The program continues to be alert to new developments emanating from scientist-originated basic research so as to properly provide the needed stimulus to hasten the field in applying this knowledge to practical use in man. The general intent of the program is to serve as a focus for all activities on antivirals conducted by Extramural, Intramural and Collaborative Research of the NIAID and to some extent the NIH.

With the advice of ad hoc committees and the Infectious Disease Branch Advisory Committee there has been a shift in emphasis toward more clinical studies. Studies currently being pursued include:

Assay: In order to support and expedite research in the area of interferon, studies were undertaken to develop more rapid, quantitative and standardized assays. A variety of parameters involved in the assay systems were studied, and information obtained did lead to a considerable reduction in the time required for the performance of these assays. A radiochemical assay has been adapted to human interferon and a mechanism for readily assaying large quantities of interferon samples obtained. Two contracts in this area have been completed. A third, which has developed a new assay based on the reduction in yield of viral neuraminidase is being continued in order to further develop this promising assay.

Standards: In an effort to better coordinate results from the different laboratories engaged in interferon research, this program has actively participated in the establishment of international interferon and interferon-inducer standards. The program is responsible for rabbit and mouse interferon and the inducer polyinosinic·polycytidylic acid standards. Studies on the lyophilization are being terminated and lyophilized standards of mouse and human interferons will soon become available. These reference reagents are made available to the scientific community through the NIAID Research Resources Branch. Through a contract on the development of specific antisera to interferon, the ASP has obtained highly specific antisera to mouse interferon. This will also be made available to the field; work is currently under way to produce anti-human interferon sera.

Production: If interferon proves effective in the treatment of human viral diseases, two methods of administration are available: the administration of exogenous interferon, produced in vitro, or endogenous interferon through the use of inducers. For the former, there is a need for the development of a technique which will provide a more efficient, safe and economically feasible method of producing large quantities of human interferon. A wide variety of

[illegible] have been tested to determine their relative capacity to produce [illegible] using a series of inducers. We have shown that there is very little [illegible] between cells in their ability to produce interferon, with human [illegible] showing some advantages. These comparative studies have been [illegible] and the program is concentrating on superinduction studies. A [illegible] to provide the mechanism for large-scale production of interferon [illegible] cell and induction system is established is progressing well. [illegible] examine the endogenous route, studies are being undertaken to [illegible] the relative merits of biological inducers to determine their potential clinical application.

Purification: If exogenous interferon is to be used in man, there is a need for purification and concentration. Work is being pursued in this area; the ultimate desirable goal of this study would be purification, chemical structure definition and synthetic manufacture.

Animal-models: In continued efforts to meet the urgent need of standardization in this field, several animal-models have been established. These are models in which the disease closely mimics a viral disease in man. Baseline data are [illegible] established using exogenous interferon and the inducer polyinosinic·polycytidylic acid (poly I·poly C). These data are then available for comparison of new antivirals as they develop. These contracts have already proven their value to the field in being able to--within a short period of time--assess the value of a new antiviral as it becomes available. These findings were accepted for publication and were well received by the editorial staff as a service to the scientific community.

Clinical Studies: The state of the art has progressed to the point where [illegible] clinical studies can be contemplated. The interferon inducer poly I·poly C has been standardized and is available for clinical studies. The availability of this material avoids variations which would be encountered in preparation of multiple lots. This inducer is currently being used in studies of herpetic keratoconjunctivitis and contemplated for use in life-threatening diseases where no therapy is available. Clinical studies are also being initiated using exogenous human leucocyte interferon in treating disseminated herpes zoster. Additional clinical studies are being contemplated assessing the relative clinical efficacy of Ara-A and IDU in herpetic encephalitis and disseminated zoster.

[illegible] contracts initiated in FY 1972 include studies on biological inducers of interferon, amplifying our clinical studies on poly I·poly C in herpetic [illegible] keratoconjunctivitis and adenovirus keratoconjunctivitis, clinical studies utilizing exogenous interferon, Ara-A and IDU in disseminated zoster and herpes encephalitis. The research sponsored by this program will continue to be an attempt to bridge the gap from scientist-originated research to practical use in man.

BAYLOR UNIVERSITY - (PH43-68-963)

Title: Evaluation of Experimental Respiratory Virus Prophylactic and Therapeutic Agents in Normal Volunteers

Contractor's Project Director: Robert B. Couch, M.D.

Project Officer: Julius Kasel, Ph.D.

Objectives: To test experimental respiratory virus vaccines and chemoprophylactic agents, and to develop and evaluate principles for development of such agents.

Major Findings: Inactivated M. pneumoniae vaccine, Lot 006004, prepared by Huntingdon Research Center was tested. The vaccine caused a high frequency of local reactions and induced appreciable antibody rises although a more potent vaccine or a booster regimen should be used in individuals with no prior antibody. Challenge studies, although at a preliminary stage, indicate that a single parenteral injection with this particular vaccine is not protective.

Adenovirus 1, 2 and 5 subunit vaccines produced by National Drug were tested both as aqueous and alum preparations. Fluid vaccines were nearly as effective in forming serum neutralizing antibody as vaccines containing alum. Adeno 5 crystalline hexon vaccine, Lot 120270, studies indicated that dosages greater than 30 mg of protein were needed for appreciable antibody titers. The Wyeth type 7 subunit vaccine studies indicated low antigen content since the antibody rise in individuals with no prior antibody was low.

The X-31 influenza recombinant derived by Dr. Edwin Kilbourne and vaccine prepared by Evens Medical Limited of England has been studied. It has surface antigens of the Hong Kong variant but the growth ability of the Λ_0 PR8 influenza strain. It was found to be as antigenic and protective as a standard Hong Kong variant (A2/Aichi) vaccine. It was also shown to induce antineuraminidase antibody. This vaccine appears to be potent enough to proceed with further evaluation.

The National Drug live attenuated parainfluenza vaccine Type 1, Lot 7286; Type 2, Log 1013; and Type 3, Lot 6806 were tested. Of the three preparations only Type 1 infected in one instance and there was unexplained illness associated with two trials of the Type 3. Therefore, these preparations do not appear to be good candidate vaccines.

Rhinovirus vaccines were examined and the individual types tested proved that live vaccines were feasible as immunogens; however, the significant developmental problems in this area - e.g. 89 rhinovirus types and difficulty of obtaining attenuation - do not warrant further work.

Significance to NIAID Programs and Bio-Medical Research: This group possesses the experience and facilities to carry out both open and closed clinical studies which are necessary for the evaluation of various vaccines in the program.

BAYLOR UNIVERSITY - (PH43-68-963) continued

Proposed Course of Project: This contract will be in effect until December 31, 1972. Renewal is anticipated.

Date Contract Initiated: June 20, 1968

Current Annual Level: $195,631

UNIVERSITY OF CALIFORNIA - DAVIS - (NIH-70-2171)

Title: Production of Interferon in Cultures of Human Cells

Contractor's Project Director: Robert S. Chang, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Develop a technique for more efficient, safe and economically feasible production of human interferon. This tissue culture expert will study a variety of human cell systems which have potential for interferon production. He will also examine the availability of amnion cell cultures to repeatedly produce interferon and define the relative costs of the different interferon-producing procedures.

Major Findings: The contractor has screened a number of human cells to date with the conclusion that lymphoid cells are no better than fibroblasts and that the human amnion seems to be the most interesting tissue. He has studied a number of variables in cell culture with no major enhancing effect. He will soon finish examining the microcarrier system to increase yield of cells and therefore interferon per ml of fluid.

Significance to NIAID Programs and Bio-Medical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found. It is expected that by the end of this contract year a wide series of cells will have been tested so as to draw some conclusions as to the best cell for production of human interferon. At that time we should be able to narrow our efforts in this area.

Proposed Course of Project: The work of this contract should be completed by the end of this contract year, when the project will be terminated.

Date Contract Initiated: June 23, 1970

Current Annual Level: (prior year support)

UNIVERSITY OF CALIFORNIA - SAN DIEGO - (NIH-70-2263)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Connor, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to evaluate the reactivity and immunogenicity of four vaccinia viruses when administered percutaneously for primary vaccination in infants and children is in progress. Six months following the primary vaccination a percutaneous challenge with the present licensed vaccine is administered. Serum antibody response are being measured by the hemagglutination inhibition and the neutralization tests. Very little of the post-challenge antibody data is available. Since this is a blind random study, a statistical analysis of the results will not be available until the end of the study. However, it appears that a primary subcutaneous immunization with a low dose of vaccinia virus results in lowered morbidity when the current licensed vaccine is administered percutaneously.

In a portion of the study which was not blind, 5 of 24 recipients of the Lederle vaccine by the subcutaneous route had swelling and erythema of the arm which lasted 2-3 days. Three of the five had temperatures. Two of the vaccinees with reactions had received the 10^3 concentration, 2 the 10^7 concentration and 1 the 10^5 concentration of vaccine. All of these five children had a family history of major allergy - not eczema. A total of 128 children who received the other three vaccines, had essentially no morbidity.

Significance to NIAID Programs & Bio-Medical Research: The recommendations of the Public Health Service and the Pediatric Society are to discontinue routine smallpox immunizations. Recent outbreaks of smallpox may influence these recommendations. It is hoped that the present collaborative study will result in information that can be used to determine a better vaccination procedure -- whether or not it is used routinely.

Proposed Course of Project: Contract was awarded through December 1972.

[illegible]: June 22, 1970

[illegible]: $[illegible],000 (18 months FY '71 funds)

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO - (NIH-71-2195)

Title: Stabilization of Polyinosinic·Polycytidylic Acid Against Serum Hydrolytic Enzymes

Contractor's Project Directors: R. Bhatnagar, Ph.D.
I. Zipkin, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Complexing polyinosinic·polycytidylic acid with various polyanious and non-antigenic proteins to render the interferon inducer more resistant to human serum hydrolytic enzymes. Ultimate goal is to render the inducer more effective and less toxic for use in man. As stable complexes are obtained, they will be produced in sufficient quantities for further testing in the animal model system.

Major Findings: The investigators have prepared complexes of poly I·poly C with DEAE-dextran, spermine and spermidine. These complexes all prove very resistant to pancreatic ribonuclease even at high concentrations of the enzyme. The data obtained indicates that complexing poly I·poly C with some of these compounds gives a product that is quite different and quite stable by chemical analysis. The current emphasis of this project is to determine the *in vivo* action of the compounds as interferon inducers.

Significance to NIAID Programs and Bio-Medical Research: Interferon remains a very promising antiviral for potential use in man. Two methods of administration are available: exogenous, prepared *in vitro*, and endogenous, through use of inducers. Of the inducers, poly I·poly C continues to have some promise. The ultimate answer as to its usefulness in humans is the degradation problem occasioned by the nucleases found in human serum. This contract is an attempt to stabilize the compound so that the dosage can be reduced without affecting and hopefully increasing its inducing abilities.

Proposed Course of Project: It is expected that the work outlined for this project should be completed by the end of this contract year, and it is intended that this contract be terminated at that time.

Date Contract Initiated: June 1971

Current Annual Level: $36,000

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO (NIH 71-2203)

Title: Development of an indirect hemagglutination test to measure antibody response to pneumococcal polysaccharide

Contractor's Project Director: Arthur J. Ammann, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Develop a standardized indirect hemagglutination (IHA) test to measure serum antibody to pneumococcal polysaccharide following natural infection and specific immunization with capsular antigen derived from serotypes of Streptococcus pneumoniae (types 1-9, 12, 14, 18, 19 and 23) 2) provide services as a central reference serological laboratory for the IHA test.

Major Findings: To determine the effectiveness of purified pneumococcal polysaccharide vaccines in eliciting an antibody response. A standard method of assay is necessary. Standardized methods have been devised for measuring serum antibodies to pneumococcal polysaccharide, types 1, 2, 3, 7, 8 and 12 with an indirect hemagglutination procedure. Human O-positive red cells are coated with purified pneumococcal polysaccharide and titrated in a semi-automated assay, using a commercially available instrument (Autotiter-Canalco). It is estimated that up to 3000 samples can be analyzed per month. A standard serum of known titer has been established from donors immunized with each of the six monovalent vaccines. Reproducible results with acceptable accuracy have been obtained with all types (1, 2, 3, 7, 8 and 12) studied thus far. During the ensuing year, a standardized test will be developed for types 4, 5, 6, 9, 14, 18, 19 and 23.

Significance to NIAID Programs and Bio-Medical Research: There is a need for improved serological tests for the diagnosis of pneumococcal disease and to assess the immune response in individuals receiving new pneumococcal candidate vaccines. Sponsoring the development of a standardized IHA test is a means of implementing this specific requirement of the pneumococcal program.

Proposed Course of Project: This contract will be reviewed and evaluated for renewal at the end of the contract year, in June, 1972.

Date Contract Initiated: June 1, 1971

Current Annual Level: $27,000 (estimate)

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO - (NIH-72-2500)

Title: Evaluation of the antigenicity of pneumococcal polysaccharide vaccines in children.

Contractor's Project Director: Arthur J. Ammann, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: This study seeks to evaluate 14 type specific monovalent pneumococcal polysaccharide vaccines in children of different age groups. It is the purpose of this study to determine the degree and duration of antibody response for each monovalent preparation. After reactivity and antigenicity have been established for each monovalent preparations, these studies will be extended to the evaluation of a polyvalent vaccine in children of the appropriate age groups.

Major Findings: Since this is a new contract, no data are reported at this time.

Significance to NIAID Programs and Bio-Medical Research: Studies of Dr. Virgil Howie and others have clearly shown that Diplococcus pneumoniae is a major etiologic agent of acute otitis media in infants and young children. There is preliminary evidence to show that children suffering with sickle cell enemia are at high risk to pneumococcal infection. Frequently many of these cases culminate in a severe form of meningitis. It is imperative to make an assessment of the response of young children to pneumococcal polysaccharides. If the antibody response is favorable, it is conceivable that a pediatric formulation of a polyvalent vaccine could be directed to reduce the excessive morbidity of these common occuring acute infectious illnesses.

Proposed Course of Project: This contract will be reviewed and evaluated before the first anniversary date, April 1973.

Date Contract Initiated: May 1, 1972

Current Annual Level: $26,870

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH - PH43-68-694)

Title: Studies of Potential Respiratory Vaccine Strains and Isolation Methods

Contractor's Project Director: Robert Magoffin, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: Develop, select and evaluate new mutant strains of parainfluenza viruses with characteristics suitable for use as potential vaccine strains.

Major Findings: Mutagenized stocks of parainfluenza virus type 3 were prepared by growing the virus in a concentration range for $10^{-3.0}$ to $10^{-4.5}$ M 5-fluorouracil. More than 600 were picked and screened for ts mutants, but no stable ts mutants were detected.

Thirty-two of 591 plaques examined (5.4% were found to replicate as well in human fetal diploid cells as in African green monkey kidney cells indicating that they were hd mutants. The rate of hd mutants produced at $10^{-3.5}$ M S-FU was 17.8% while at $10^{-3.0}$ and $10^{-4.5}$ M the mutational rate was 2.3 and 2.7% respectively.

Significance to NIAID Program and Bio-Medical Research: Because of the large amount of lower respiratory tract disease in infants caused by the parainfluenza viruses, there is a need for live attenuated parainfluenza vaccines.

Proposed Course of Project: The contract was terminated on June 30, 1972.

Date Contract Initiated: February 1, 1968

Current Annual Level: Prior year support

UNIVERSITY OF SOUTHERN CALIFORNIA - (NIH-72-2506)

Title: Evaluation of H. influenzae type b, and meningococcal (group A and C) vaccines in infants and children.

Contractor's Project Director: Paul F. Wehrle, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: Direct and coordinate studies to determine optimal immunization schedules for H. influenzae type b PRP vaccine and group A and group C meningococcal vaccines in very young children (ages 1-3) and infants less than 12 months of age. Basic variables to be explored include: reactivity, antigenicity of each candidate vaccine dose (polysaccharide concentration), route of administration, time interval between primary immunization and the booster effect accompanying a secondary immunization.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: Epidemiological studies have shown the major protion of bacterial meningitis of young children and infants is due to H. influenzae and group A and C Nasseria meningitidis. If clinical studies show the young infant can respond satisfactorily to immunization with purified polysaccharide vaccines, it may be possible to reduce the prevalence of these serious infectious diseases of the central nervous system by an immunoprophylactic approach.

Proposed Course of Project: This contract will be reviewed and evaluated before the first anniversary date, April 1973.

Date Contract Initiated: April 1972

Current Annual Level: $60,000

UNIVERSITY OF CHICAGO - (PH43-68-83)

Title: Immunization of humans with group A streptococcal proteins

Contractor's Project Director: Albert Dorfman, M.D., Ph. D.

Project Officer: Roger Cole, M.D.

Objectives: 1) Conduct a challenge study in a panel of immunized and non-immunized volunteers with type 1 streptococci. 2) Test bivalent or trivalent M-protein preparations for immunogenicity and extend monovalent testing of M-protein of types 1, 3 and 6. 3) Evaluate the booster effect of M-protein in previously immunized infants in whom the levels of bactericidal antibody have disappeared.

Major Findings: During the past year a protective study of M-protein was initiated in adult volunteers immunized with three monthly vaccine doses according to the following protocols: ten subjects received subcutaneous injections of APM-1 (85 ug of M-1 protein combined with 2.5 ug aluminum hydroxide, per dose); nine individuals received soluble M-protein sprayed topically in the nasopharynx (0.5 ml containing 200 ug M-protein, per dose); nine subjects received aluminum hydroxide placebo injections; and six received no inoculations. All subjects were infected with live type 1 streptococci in the pharynx with a swab dipped into a culture containing approximately 10^6 colony units per ml. Obvious clinical signs of infections, confirmed by positive throat cultures, and significantly elevated white cell counts and/or fever were observed in 11 of the 15 control subjects. Six of the 19 immunized subjects exhibited clinical symptoms of infection; the distribution of susceptibility was equal between the parenterally and intranasally immunized groups. It is anticipated an additional 30 subjects will be studied this year.

Eleven subjects between the ages of 5 and 13 years received a divalent intranasal immunization with soluble M-protein, types 1 and 3. The schedule consisted of three monthly doses of 100 ug of each serotype sprayed into the nasopharynx. Four of these subjects exhibited serum antibodies 30 post-immunization; 2 subjects produced antibodies to both serotypes. Nasal washings were collected from seven of these children. However, no antibodies could be detected in the secretions.

Significance to NIAID Program and Bio-Medical Research: Streptococcal disease and its sequelae, namely rheumatic fever and glomerulonephritis are significant health problems. An effective safe vaccine that reduces the prevalence of these illnesses would be of great importance.

Proposed Course of Project: This contract has been funded through May 22, 1971

Date Contract Initiated: January 1968

Current Annual Level: $83,800

CHILDREN'S HOSPITAL MEDICAL CENTER, HARVARD MEDICAL SCHOOL (NIH 71-2196)

Title: Vaccine evaluation and surveillance studies with Hemophilus influenzae type b

Contractor's Project Director: David Smith, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Provide services of a reference laboratory for the microbiological identification and serotyping of Hemophilus influenzae isolated from clinical cases of meningitis and acute otitis media 2) develop a radioimmunoassay to measure antibody response to H. influenzae type b infection and active immunization with polyribose (PRP) vaccine 3) conduct vaccine testing in children with PRP antigen to determine safety and dose response data 4) prepare at least 2 gms purified H. influenzae type b (PRP) antigen for final container packaging.

Major Findings: The contractor has produced the required 2 gms of purified H. influenzae type b (PRP) antigen material. Through a subcontract with the Massachusetts Biologic Laboratory, 648 vials of PRP vaccine at a concentration of 200 ug/ml were packaged and satisfactorily passed tests for sterility, general safety and identity. This vaccine, designated lot #5, is now scheduled for clinical evaluation by three separate contractors in young children and infants under the age of 12 months. More than 236 strains of H. influenzae isolated from the middle ear of children less than 3 years of age with otitis media have been studied. Results indicate that about 90% of the strains are non-typable by one of three methods (agglutination, direct immunofluorescence and hemagglutination inhibition). Approximately 10% of the isolates from middle exudate were identified as type b. Satisfactory progress has been made in the development of a standardized radioimmunoassay (RIA) for measuring type specific antibody to H. influenzae. An endogenous labeled PRP antigen serves as a standard. Manipulation of cultural conditions have permitted a significant increase in specific activity using C^{14}. The radioactivity of this preparation is completely bindable by standard anti-PRP sera and the binding can be completely blocked by an excess of unlabeled PRP, indicating a satisfactory isotopic purity. Thus at the present stage of development, the radioimmunoassay has a sensitivity of detecting anti-PRP antibody 30 fold greater than the passive hemagglutination test. More than 100 infants and children, ranging in age between 6 to >48 months have been immunized with varying doses (1, 5 and 25 ug) of PRP vaccine. Assessment of the antibody response by the RIA and PHA are in progress. To date there have been no local or systemic reactions, including fever.

Significance to NIAID Programs and Bio-Medical Research: Improved methods are needed for microbiological and serological diagnosis of H. influenzae type b infection of children. A highly sensitive procedure such as the RIA is required to measure antibody response to natural disease prototype vaccines used in immunization studies. An effective vaccine is needed to give high levels of protection against H. influenzae infections.

Proposed Course of Project: To be reviewed and evaluated prior to end of current contract year.

Date Contract Initiated: June, 1971

Current Annual Level: $110,000 (Estimate)

CHILDREN'S HOSPITAL RESEARCH FOUNDATION, COLUMBUS, OHIO (NIH-69-2062)

Title: Rhinovirus Reference Center

Contractor's Project Director: Vincent V. Hamparian, Ph. D.

Project Officer: Albert Kapikian, M.D.

Objectives: To compare by serological methods suitable candidate prototype rhinoviruses received from collaborating laboratories throughout the world in order to define the number of prevalent serotypes and to allow an agreement to be reached by interested workers on a final numbering system.

Major Findings: A total of 89 rhinoviruses were defined as prime strains at the close of phase II of the rhinovirus typing program. During the past year, the relationship's among 11 candidate prototype rhinoviruses and 5 numbered rhinoviruses were clarified. Some of the viruses were shown to be new prime strains, some were the same as previously numbered prime strains and some were shown to have low grade to moderate relationships to other rhinoviruses. Those showing cross reactivities may be designated as subtypes.

Fifteen to 25 candidate prototype rhinoviruses are expected for the third phase of the collaborative rhinovirus program. This phase will be completed during the coming year.

Significance to NIAID Program and Bio-Medical Research: Rhinoviruses are the major cause of the "common cold". In order to find prophylactic methods for combating the "common cold" it is necessary to know the causative agent.

Proposed Course of Project: To continue through March 31, 1973

Date Contract Initiated: May 1, 1969

Current Annual Level: $33,700

RESEARCH FOUNDATION OF CHILDREN'S HOSPITAL, WASHINGTON, D.C. - (NIH-71-2091)

Title: Evaluation of Respiratory Virus Vaccines in Infants and Children

Contractor's Project Director: Robert Parrott, M.D.

Project Officer: Robert Chanock, M.D.

Objectives: 1) To study presumably attenuated viruses as potential vaccines. 2) To study factors in the resistance to and pathogenesis of illness resulting from natural infection with respiratory viruses, particularly RS and parainfluenza viruses. 3) To study the "paradoxical" effect following intramuscular injection of inactivated RS vaccine.

Major Findings: TS-1 Mutant RS-A2 virus was administered to 26 infants and children 9 months to 6 years of age and infected all of them. No illness accompanied TS-1 infection in individuals who had prior experience with RSV. Infection in infants without prior RSV experience resulted in mild afebrile coryza with no systemic signs.

Virus from 19 of the 22 who shed virus showed the temperature sensitive characteristic. However, "revertant" virus was found in 3 vaccines with late onset of shedding and lower levels of shedding than in infants undergoing primary infection.

RSV neutralizing activity has been found in the secretions of some infants who had no prior RSV infection. This activity is found in a heavy sucrose density fraction where IgM would be expected; however, the activity could not be correlated with any immunoglobulins.

In follow-up studies of children who previously received inactivated RS virus vaccine it appears that an untoward "paradoxical" effect has either dissipated 5 years after administration of vaccine or the children now have sufficient local immunity to prevent significant reinfection.

The absence of RSV illness during an RSV outbreak in 34 infants or children who received RS-A2, 26°C or TS-1 mutant prior to the outbreak suggests the possibility that the resistance of these infants to RSV was enhanced by the administration of the potential vaccines.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus and the parainfluenza viruses are the major known causes of severe respiratory illness in young children.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at the end of the contract year (December 1972).

Date Contract Initiated: December 7, 1971. This contract was previously number PH43-62-473.

Current Annual Level: $141,000

UNIVERSITY OF COLORADO - (PH-43-62-477)

Title: Respiratory Virus Vaccine Evaluation and Surveillance

Contractor's Project Director: Kenneth McIntosh, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To clinically evaluate in selected populations the following vaccines: live respiratory syncytial virus, meningococcal and *Hemophilus influenzae* type b polysaccharides, and inactivated parainfluenza virus.

Major Findings: A study was initiated to assess optimal immunization procedure for meningococcal A or C vaccine in 77 children (ages 5-11) at the Colorado State Home and Training School. The primary immunizing dose was 50 ug. Twenty-six weeks later, all of the children in each group were given a booster dose of 50 ug. Response of children to the six-month booster dose was not remarkable (only 40% experienced a significant titer increase by the IHA test). In a second study, 22 normal children (age 1-4 years) were given 20 ug or 50 ug group C vaccine. All children had a rise in IHA antibody but levels were variable.

Preliminary tests with RSV-ts-1 mutant are currently being evaluated. There is some evidence of vaccine virus spread. The take rate is high in children under five (7 of 7) but low in older children (4 of 15). This proposal plans to continue to study RSV vaccines in groups of 20-50 children at the National Jewish Hospital, since respiratory syncytial virus infection is particularly prevalent in asthmatic children.

Significance to NIAID Programs and Bio-Medical Research: Provides important pediatric and adult populations for vaccine evaluation.

Proposed Course of Project: The contract was terminated on June 19, 1972.

Date Contract Initiated: June 1, 1962

Current Annual Level: Prior year support

UNIVERSITY OF COLORADO - (NIH-70-2264)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: C. Henry Kempe, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to evaluate the reactivity and immunogenicity of four vaccinia viruses when administered percutaneously for primary vaccination in infants and children is in progress. Six months following the primary vaccination a percutaneous challenge with the present licensed vaccine is administered. Serum antibody response are being measured by the hemagglutination inhibition and the neutralization tests. Very little of the post-challenge antibody data is available. Since this is a blind random study, a statistical analysis of the results will not be available until the end of the study. However, it appears that a primary subcutaneous immunization with a low dose of vaccinia virus results in lowered morbidity when the current licensed vaccine is administered percutaneously.

Significance to NIAID Programs & Bio-Medical Research: The recommendations of the Public Health Service and the Pediatric Society are to discontinue routine smallpox immunizations. Recent outbreaks of smallpox may influence these recommendations. It is hoped that the present collaborative study will result in information that can be used to determine a better vaccination procedure -- whether or not it is used routinely.

Proposed Course of Project: Contract was awarded through December 1972.

Date Contract Initiated: June 15, 1970

Current Annual Level: $62,900 (18 months FY '71 funds)

UNIVERSITY OF COLORADO - (NIH-72-2501)

Title: Clinical evaluation of a temperature sensitive mutant respiratory syncytial virus vaccine in asthmatic children; and evaluation of meningococcal (groups A and C) vaccines and Hemophilus influenzae type b PRP vaccine in children for safety and antigenicity.

Contractor's Project Director: Kenneth McIntosh, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: Live virus vaccines with major emphasis on a nutagen-produced temperature-sensitive RSV vaccine will be tested in closed populations of children to ascertain infectivity, lack of virulence and production of antibody.

Optimal immunization schedules will be explored by the adminitration of Hemophilus influenzae type b (PRP) vaccine and group A and C meningococcal vaccines in children and infants. Variables to be studied for each of the bacterial vaccines include safety and antigenicity. Furthermore, as assessment will be made concerning route of administration, concentration of dose and the role of a secondary immunization in provoking a booster antibody response. Serological response for each bacterial vaccine will be measured by a standardized radioimmunoassay procedure.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: Asthmatic children contracting RSV infection experience a high incidence of acute wheezing attacks and pronounced pulmonary changes on X-ray examination. These studies will establish the role of an attenuated RS vaccine to induce immunity in this group of allergic children.

Studies concerned with the administration of H. influenzae type b, meningococcal (group A and C) to children are directed to developing effective immunization programs for H. influenzae and meningococcal meningitis of young children and infants.

Proposed Course of Project: This contract will be reviewed and evaluated before the next anniversary date, June 1973.

Date Contract Initiated: April 4, 1972

Current Annual Level: $79,400

UNIVERSITY OF CONNECTICUT - (NIH-72-2502)

Title: Serological response of infants to meningococcal group A and C polysaccharide vaccines.

Contractor's Project Director: Martha Lepow, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: The purpose of this study is to determine the serological response of 3 month old infants to immunization with meningococcal group A and C polysaccharide vaccines. This project will undertake to determine the response of infants to different doses of meningococcal vaccine administered at different ages. A second objective of the project will be a study of the prevalence of meningococci in the upper respiratory tract of approximately 500 first grade and 500 fourth grade school children.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: The peak incidence of meningococcal disease in children is between 6 to 24 months of age. Therefore, it is essential to determine the optimal dose, frequency, and time of immunization in order to be able to protect this most susceptible age group by immunization.

Proposed Course of Project: This contract will be reviewed and evaluated before the first anniversary date, April 1973.

Date Contract Initiated: April 1973

Current Annual Level: $32,805

CORNELL UNIVERSITY MEDICAL COLLEGE (NIH-70-2065)

Title: Study of the antigenicity and immunogenicity of Mycoplasma pneumoniae vaccines and development of an animal model system for the study of respiratory syncytial virus

Contractor's Project Director: Laurence Senterfit, Sc.D.

Project Officer: Robert Purcell, M.D.

Objectives: 1) Collaborate with Drs. Somerson (Ohio State University) and Hamparian (Children's Hospital, Columbus, Ohio) in the production of inactivated M. pneumoniae vaccine by performing the required animal studies 2) study the immunogenicity and antigenic structure of temperature sensitive (ts) mutants of M. pneumoniae 3) complete the development of methods for the qualitative and quantitative measurement of antibody to specific determinants of the Mycoplasma membrane 4) conduct serological testing of sera from clinical trials with HRC and Ohio State vaccines 5) to study the adjuvant effect of polynucleotides on viral, mycoplasmal and bacterial vaccines.

Major Findings: Due to a continuing bacterial contamination (vibrio percu-lans) problem in the production area at HRC, it was deemed advisable to change the production facility to Ohio State University where it is now under the direct management of Drs. Somerson and Hamparian. Production has been satisfactory under this arrangement and it is anticipated that at least 10 liters of a high potency vaccine will be completed by July, 1972.

All sera from clinical trials of the HRC M. pneumoniae vaccine have been tested by the contractors utilizing the Tetrazolium Reduction Inhibition (TRI) test and the Complement Fixation (CF) test, with lipid haptene glycolipid antigen. Results indicate that the vaccine produces significant levels of antibody in 80 to 100% of the volunteers.

Studies have been initiated using the radioimmuno precipitation procedure and the immunoelectro osmophoresis as sensitive methods for the determination of antibody to M. pneumoniae. Concentrated RS viruses have been prepared by precipitation with polyethylene glycol and sucrose gradient centrifugation. At present, the preparations of virus can not be internally labeled to a degree that would be useful as a reagent for the detection of low levels of antibody by the radioimmuno precipitation procedure. There is evidence to suggest that the problem can be overcome by using labeled amino acid filtration techniques.

There appears to be an unequivocal adjuvanting effect of Poly I:C with M. pneumoniae, parainfluenza and respiratory syncytial inactivated vaccines. This effect is quite dramatic and occurs at least with M. pneumoniae vaccine by both the parenteral and intranasal route of administration.

Significance to NIAID Programs and Bio-Medical Research: There is a need for an improved animal model system in which the serological response to potential inactivated M. pneumoniae vaccines will more closely parallel the

human [illegible]. Such an animal model will aid in the selection of a more potent Mycoplasma vaccine.

Proposed Course of Project: The contract will be reviewed and evaluated prior to the anniversary date, January, 1973

Date Contract Initiated: January 18, 1970

Current Annual Level: $52,100

UNIVERSITY OF FLORIDA COLLEGE OF MEDICINE - (NIH-70-2170)

Title: Production of Human Interferon

Contractor's Project Director: George E. Gifford, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Develop a technique for a more efficient, safe and economically feasible method of producing human interferon. This contractor will concentrate his efforts on the ability of continuous and transformed cell lines derived from different human tissues to produce human interferon.

Major Findings: The contractor has screened a variety of human cell types under several culture conditions and infected with various viruses at a number of different multiplicities for the ability to produce interferon. He has concentrated on monolayer and suspension cultures of cells derived from the human placenta, whole perfused placenta, a continuous line of amnion cells (U cells), and four human embryonic lung lines. The investigator has ascertained that (1) amnion cells infected with NDV yield the most interferon units; (2) aging in culture for 2 to 3 weeks after initiation enhances interferon yield; (3) trypsinization decreases interferon yields; (4) too high a multiplicity of infection (NDV) can decrease interferon yields, but a wide range of multiplicities is effective for yielding high interferon levels in this system; (5) poly I·poly C requires the presence of DEAE-dextran to induce reasonable levels of interferon in human amnion cells; (6) the serum concentration was not of major concern; (7) most interferon is produced within 24 hours after exposure to the inducer. Some evidence is available that release of lysosomal enzymes is related to low yields of interferon production.

Significance to NIAID Programs and Bio-Medical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 23, 1970

Current Annual Level: $20,500

FLOW LABORATORIES - (PH-43-68-1309)

Title: Operation of Virus and Tissue Culture Laboratories

Contractor's Project Director: Raymond V. Gilden, Ph.D.

Project Officer: Robert M. Chanock, M.D.

Objectives: (1) To perform research and development activities in support of the Laboratory of Infectious Diseases (LID), NIAID respiratory virus and mycoplasma research program; (2) to develop and prepare tissue cultures for LID's program and for the program described in (1).

Major Findings: Respiratory Syncytial Virus - Following exposure to ethylmethane sulfonate (EMS) and proflavine sulfate, a total of 4 ts mutants (2 from each mutagen treatment) were isolated which in in vitro and in vivo assays appear promising as potential vaccine candidates. A multi-step, cold-adapted mutant has been selected from a WI-38 adapted line of RS virus which possesses the ts property.

Parainfluenza Viruses - Following exposure of a 35°C triply-plaque-purified-cloned population of BEK grown type 3 virus to 5-fluorouracil, two ts mutants from the treated population and one ts mutant from the control, untreated populations were isolated. Since it is not known whether the mutants recovered from the 5-FU treated population were induced or spontaneous, all type 2 and type 3 virus strains (AGMK, WI-38, and/or BEK grown) were triply-plaque-purified at 39°C in their respective tissue culture systems and cloned populations prepared for future mutagenesis studies. Multi-step, cold-adapted mutants have been selected from the AGMK and WI-38 grown strains of type 3 virus which possess the ts property.

Experimental Virus Vaccines - Eight pools (approximately 2 liter amounts) of RS virus (TS-1 [2x], TS-3, TS-4, TS-5, TS-6, TS-7 and parent) have been produced, of which 5 have been satisfactorily safety tested to date. Two pools (approximately 2 liter amounts) of type 1 parainfluenza virus (selected mutant and parent) have been produced and satisfactorily safety tested. Eleven different pools of influenza A virus and one pool of type 1 para-influenza virus prepared at the LID have been satisfactorily safety tested.

Significance to NIAID Programs and Bio-Medical Research: This contract provides unique tissue cultures essential to various research programs of the LID. It also provides vaccine production and/or safety testing of vaccine produced by LID or by other IDB contractors. In addition, genetic mutants suitable for the preparation of parainfluenza viruses and respiratory syncytial virus vaccines are being developed.

Proposed Course of Project: Contract will be reviewed and evaluated at the end of the current contract period.

Date Contract Initiated: June 23, 1968.

Current Annual Level: $400,500

GEORGETOWN UNIVERSITY - (PH-43-67-745)

Title: Studies of Local Antibody in Respiratory Viral Immunity

Contractor's Project Director: Joseph Bellanti, M.D.

Project Officers: Robert Chanock, M.D., and Daniel I. Mullally, M.D.

Objectives: 1) To complete studies of humoral and local antibody in children and adults naturally or experimentally infected with respiratory syncytial virus (RSV). 2) To complete the study of M. pneumoniae antibody in serum and nasal secretions using a radioactively labeled phospholipid hapten. 3) To develop a more sensitive test for the presence of nasal antibody to influenza virus in secretions, using a radioactively labeled influenza virus preparation.

Major Findings: Studies of serum and local antibody responses following infection with Mycoplasma pneumoniae indicate that the predominant antibody in serum is of the gamma G variety. Antibody in respiratory secretions cannot be regularly detected using conventional techniques. Early results indicate that the use of radio-binding employing a radioactive C^{14} glycolipid hapten appears feasible for the measurement of antibody in situations where greater sensitivity is needed.

Studies of respiratory syncytial virus antibody in nasal secretions and sera employing radioimmunodiffusion techniques utilizing C^{14} labeled RS virus have continued. The work includes the identification of gamma E neutralizing activity in nasal secretions by selective absorption employing specific anti-gamma E antibody.

Significance to NIAID Programs and Bio-Medical Research: Investigations in this program have produced some very promising leads that local antibody (IgA) in respiratory secretions is of paramount importance in the development of immunity to some respiratory illness. Volunteers receiving inactivated vaccines parenterally do not usually develop nasal antibody and can, therefore, be infected. Patients with natural infection for the most part develop nasal antibody in addition to serum antibody. These observations suggest that the ability to express antibody in nasal secretions might be an important consideration in devising methods of immunoprophylaxis against respiratory disease.

Proposed Course of Project: Contract was terminated on April 18, 1972.

Date Contract Initiated: April 19, 1967

Current Annual Level: Prior year support

GEORGETOWN UNIVERSITY MEDICAL SCHOOL - (NIH-70-2186)

Title: Polyinosinic acid-polycytidylic acid complexes: Studies on the Variables in the Formation of Stable and Biologically Active Complexes

Contractor's Project Director: T. Sreevalsan, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Study the variables in the procedure for formulating the double-stranded polyinosinic·polycytidylic (In·Cn) acid in order to obtain a stable, reliable, potent and less toxic preparation of In·Cn.

Major Findings: Various studies were undertaken in an attempt to avoid the extreme variability in antiviral activity seen in the various cell lines using various preparations of poly I·poly C. These studies have resulted in a formulation procedure which yields a more active compound than has been previously described. However, the results obtained thus far do not explain the basis for the variability of the poly I·poly C preparation. There is some suggestion that this variability may be due to the state of the physiology of the cells in culture. The current studies will address themselves to the relationship of the molecular weight of poly I·poly C to its antiviral activity. The investigations carried out thus far will be useful in the future for formulation of this interferon inducer in terms of obtaining a repeatable, reliable product.

Significance to NIAID Programs and Bio-Medical Research: The current method of formulating In·Cn, although adequate, does not consistently result in a reliable product. There is a need for a method which would yield a consistent and less toxic In·Cn. It is expected that this study will yield such a method.

Proposed Course of Project: It is expected that the work on this project will be completed by the end of the current contract year and is to be terminated.

Date Contract Initiated: June 26, 1970

Current Annual Level: (prior year support)

INDIAN HEALTH SERVICE, HEALTH SERVICES AND MENTAL HEALTH ADMINISTRATION
(Interagency Agreement #70-A-11)

Contractor's Project Director: George E. Bock, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: To conduct clinical and laboratory surveillance among Navajo Indians living in the Gallup Service Area and establish an incidence rate for pneumococcal pneumonia, otitis media and meningitis in preparation for a pneumococcal polysaccharide vaccine field trial.

Major Findings: In collaborations with the University of New Mexico (Project Director, Dr. Robert Oseasohn) a highly qualified epidemiological team was established at the Gallup Indian Public Health Service Hospital, Gallup, New Mexico. A feasibility study was conducted to determine if the populations living within the confines of the Gallup Service Unit were a high risk group with sufficient natural disease to warrant a vaccine field trial. Analysis of the data gathered at the Gallup Hospital between July-December 1971 revealed a total of 304 cases of pneumonia, (110 cases of in-patient pneumonia and 194 cases of outpatient pneumonia). A crude incidence rate for total pneumoniae in individuals 15 years or older is estimated at 20 cases/1000 per year. Pneumococcal isolates were recovered from 81% of the total cases of pneumonia. Seventy-one percent of the pneumococcal isolates consisted of serotypes (1-9, 12, 14, 18, 19 and 23).

Selected studies on acute otitis media were conducted on Navajo children under the age of 5 years. Pneumococci (types 5, 6, 7, 9, 14, 15, 19, 20 and 23) were recovered from 51% of the middle ear exudates. It is apparent that the subgroup of Navajo Indians living in the Gallup Service Unit has an exceedingly high incidence of pneumococcal disease. Plans are underway to structure a field trial in a thin segment of the population to show efficacy of a candidate polyvalent pneumococcal vaccine.

Significance to NIAID Programs and Bio-Medical Research: To demonstrate efficacy of prototype pneumococcal polyvalent vaccines populations with significantly high incidence rates of pneumococcal disease are required. This particular population fulfills this basic requirement and would stand to benefit immensely from an immunoprophylactic agent of this nature.

Proposed Course of Project: This interagency agreement will be reviewed and evaluated for renewal at the end of the 1972 fiscal year.

Date Agreement Initiated: July 1969

Current Annual Level: $78,000

UNIVERSITY OF KENTUCKY - (NIH-70-2265)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: Abram S. Benenson, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to evaluate the reactivity and immunogenicity of four vaccinia viruses when administered percutaneously for primary vaccination in infants and children is in progress. Six months following the primary vaccination a percutaneous challenge with the present licensed vaccine is administered. Serum antibody response are being measured by the hemagglutination inhibition and the neutralization tests. Very little of the post-challenge antibody data is available. Since this is a blind random study, a statistical analysis of the results will not be available until the end of the study. However, it appears that a primary subcutaneous immunization with a low dose of vaccinia virus results in lowered morbidity when the current licensed vaccine is administered percutaneously.

Significance to NIAID Programs & Bio-Medical Research: The recommendations of the Public Health Service and the Pediatric Society are to discontinue routine smallpox immunizations. Recent outbreaks of smallpox may influence these recommendations. It is hoped that the present collaborative study will result in information that can be used to determine a better vaccination procedure -- whether or not it is used routinely.

Proposed Course of Project: Contract was awarded through December 1972.

Date Contract Initiated: June 1970

Current Annual Level: $65,000 (18 months FY '71 funds)

KAISER PERMANENTE MEDICAL GROUP OF SAN FRANCISCO - (NIH-72-2513)

Title: Epidemiological study of pneumococcal disease

Contractor's Project Director: Marvin A. Freid, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Based on appropriate clinical examination, including chest roentgenograms, determine a firm incidence rate for pneumococcal pneumonia in subscribers in respect to age, sex, pre-existing disease and place of residence. 2) Determine the prevalence of pneumococcal serotypes responsible for pneumococcal disease by appropriate bacteriological examinations of blood sputum, naso-pharyngeal secretions or other body fluid specimens.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: Currently there is much interest in the prevalence of pneumococcal disease in a large open population. There is preliminary evidence to suggest subscribers of this medical group experience a relatively high incidence of disease. The program is sponsoring an intensive epidemiological study of pneumococcal disease in this population with ultimate aim of conducting a large field trial to show efficacy of a pneumococcal polyvalent vaccine.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at the end of the contract year.

Date Contract Initiated: March 1, 1972

Current Annual Level: $105,868

LEE-MAC - (NIH-71-2198)

Title: Production of Interferon in a Unique Cell Culture System

Contractor's Project Officer: Thomas McCoy, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: If exogenous interferon proves to be effective in prophylactic and/or therapeutic use in man, an economically feasible method of producing interferon is needed. During the first year of this contract a feasibility study was performed to determine whether a unique cytogenerator could be used for this purpose. The current contract year is to amplify these studies.

Major Findings: A variety of alterations of media, pH, and manipulation of the cytogenerator have been successful in demonstrating the feasibility of producing large masses of diploid cells in this system. These studies have been successful in obtaining 3×10^8 viable cells in a volume of 200 milliliters. This is equivalent to the amount of cells produced in 75 completely confluent 250 ml tissue culture flasks. Studies are currently under way to examine the various factors which need to be clarified in order to obtain the maximum yield of interferon. This is particularly important since in vitro large population densities of cells in the cytogenerator do not necessarily function in the same manner as those in conventional monolayers. These aspects include cell selection, techniques of challenging cells, factors affecting cell response, and multiple use of inducers.

Significance to NIAID Programs and Bio-Medical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available: exogenous, prepared in vitro, and endogenous, through use of inducers. There is currently no successful method of economically producing large quantities of human interferon produced in cells other than human leucocytes. This contract is an attempt to determine whether a unique cytogenerator can overcome this obstacle.

Proposed Course of Project: To be reviewed and results evaluated. If the cytogenerator in producing large quantities of interferon, attempts will be made to scale up. Because this is a fixed price contract, it must be terminated on June 20, 1972. A new contract will be initiated if subsequent work is to be done.

Date Contract Initiated: June 1971

Current Annual Level: $52,000 (estimated)

ELI LILLY AND COMPANY - (PH43-68-1280)

Title: Production of specific immunogenic polysaccharide vaccines from selected serological types of streptococcus pneumoniae

Contractor's Project Director: Robert S. Baker, Ph. D.

Project Officer: Richard E. Horton, M.D.

Objectives: Complete production of at least 25 gms of each of pneumococcal polysaccharide types (1-9, 12, 14, 18, 19 and 23).

Major Findings: Bulk antigen meeting both chemical and immunological specifications of the contract have been produced for types 1-9, 12, 14, 18, and 19. Cultivation and purification procedures have been devised on a pilot scale for type 23. Final packaging and testing for sterility, identity and general safety in animals have been accomplished for monovalent vaccine types 1-9, 12, 14, 18 and 19. Formulation of two candidate hexavalent vaccines of types 1, 3, 4, 7, 8, and 12 have passed tests for safety, purity and identity. Preliminary clinical trials with all monovalent and polyvalent vaccines show that these vaccines are free of any serious local or systemic reactions.

Significance to NIAID Programs and Bio-Medical Research: Vaccines forthcoming from this contract represent a highly significant segment of activity of the overall pneumococcal vaccine program to determine the efficacy of a polyvalent vaccine to reduce the rising incidence of pneumococcal disease.

Proposed Course of Project: It is anticipated the contractor will complete the assigned work task by December 1972.

Date Contract Initiated: June 1968

Current Annual Level: $51,400

MELOY LABORATORIES (NIH 71-2097)

Title: Maintenance of animal holding facility

Contractor's Project Director: John E. Verna, Ph. D.

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: To provide an animal holding facility, under isolated conditions, for the study of hepatitis in various primates, chiefly the rhesus monkey; to assist the project officer with animal observations, autopsies, collection of tissue specimens and histopathology.

Major Findings: During the past year, caesarean-derived rhesus monkeys have been inoculated with hepatitis B serum (subtype ad) obtained from a carrier of HB Ag with chronic hepatitis. Using the most sensitive tests for HB Ab detection (radioimmunoprecipitation techniques and passive hemagglutination) and HB Ag (solid-phase radioimmunoassay) both antibody and antigen were detected in the rhesus monkey.

Hepatitis B serum containing hepatitis B antigen seroconverted 5 out of 6 rhesus monkeys within two to three weeks after inoculation. Passage of late serum from these seroconverted rhesus monkeys to other rhesus monkeys resulted in seroconversion in three out of four cases in one passage series.

The first large-scale studies (approximately 60 rhesus monkeys) have just been initiated. These experiments will include an infectivity titration of a newly prepared fifth rhesus monkey. Passage pool as well as preliminary experiments on the resistance of the agent to heat and chemicals and the establishment in the rhesus monkey of an infectious pool of hepatitis B virus, subtype ay.

Significance to NIAID Program and Bio-Medical Research: These animal model studies are a necessary and integral part of the NIAID Collaborative Hepatitis Project, as they will yield basic knowledge on the characteristics of the agent of hepatitis B.

Proposed Course of Project: The contract has been renewed through December 31, 1972.

Date Contract Initiated: June 23, 1967

Current Annual Level: $266,000

UNIVERSITY OF MICHIGAN - (PH-43-66-462)

Title: The Epidemiology of Respiratory Disease in a Total Natural Community and Evaluation of Immunizing Agents

Contractor's Project Director: Arnold Monto, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To provide epidemiological data on respiratory diseases in the Tecumseh study area. To conduct vaccine trials in institutions, schools and/or clinics in the area; selection of the location will depend on the composition of the study group required to answer the questions posed by the particular vaccine.

Major Findings: Children age 2-6 years were inoculated with varying amounts of group C meningococcal polysaccharide vaccine. Doses of 5, 25 and 50 mcg were found less satisfactory in eliciting antibody than 100 or 250 mcg. Antibody response was directly related to age. Children in 3 of the vaccine groups were reinoculated 8 months later with 50 mcg of both group A and group C vaccines. Booster responses occurred in all groups and those who received the highest doses originally still had the greatest amount of group C antibody. All responded to group A vaccine. The hemagglutination method did not offer sufficient sensitivity in detection of antibody while the radioimmunoassay procedure was satisfactory. Even using this sensitive technique, the response in children was of lower magnitude than that found in adults given smaller doses of vaccine. Live parainfluenza virus type 1, prepared by National Drug Company, was administered intranasally to children aged 6-14 years. No illnesses occurred. Five out of 20 had a three-fold or greater rise in serum neutralizing antibody. This vaccine is now being administered to children aged 2-6 years to see if it is safe and antigenic in that age group.

Significance to NIAID Program and Bio-Medical Research: Provides a natural open population for a vaccine study.

Proposed Course of Project: The contract was terminated on June 30, 1972.

Date Contract Initiated: April 4, 1966.

Current Annual Level: $52,000

MT. SINAI SCHOOL OF MEDICINE - (NIH-70-2129)

Title: Purification of Human Interferon

Contractor's Project Director: Panayotis G. Katsoyannis, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Utilizing crude interferon supplied through the NIH, this group will undertake the purification of human interferon. The first year was used in developing techniques toward a more purified material than is currently available. This contract will continue purification studies for the dual purpose of applying the product to man and eventually synthesizing the compound.

Major Findings: Several modes to achieve a preliminary enrichment of the material have been explored. These were: precipitation with ammonium sulfate, and adsorption to and elution from Doucil-25, zeolite, and Amberlite IRP-64. Ammonium sulfate precipitation caused too great a loss of biological activity to make it worthwhile; zeolite proved effective. Material with a protein concentration of 1.5 mg/ml and a specific activity of 24,000 U/mg was prepared from 100 ml batches of crude interferon using these preliminary techniques. Electrofocusing techniques are being undertaken. This group has also successfully perfected a quick assay (24 hours) for the various fractions involved.

Significance to NIAID Programs and Bio-Medical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found. This interferon must then be purified to make it safe for man. If the compound could be synthesized, it could be made available at a reduced cost.

Proposed Course of Project: If this work were to be successful, it would require a highly efficient interrelationship of two departments which would need to be highly compatible. Because of a breakdown in the efficiency of the relationships and because of Dr. Sonnabend's returning to England, it was decided that this contract should be terminated and the work continued at the NIH intramural facilities.

Date Contract Initiated: June 8, 1970

Current Annual Level: (prior year support)

MOUNT SINAI SCHOOL OF MEDICINE - (NIH-71-1371)

Title: Arrange and Conduct three Conferences on Influenza

Contractor's Project Director: Edwin D. Kilbourne, M.D.

Project Officers: Julius A. Kasel, Ph.D. and Martha J. Mattheis, M.A.

Objectives: To arrange and conduct a series of conferences (about 3 per year) on the genetics of influenza virus and new approaches to the control of influenza.

Major Findings: Two workshops entitled "Influenza Virus Polypeptides and Antigens" and "Immunologic Methodology in Influenza Diagnosis" have been held. Summaries of the workshops are being published in the Journal of Infectious Diseases.

A third workshop entitled "Specific Immunity in Influenza" will be held during this contract year. The summary of the meeting will be published.

Significance to NIAID Program and Bio-Medical Research: It is predicted that another pandemic of influenza will occur during the period 1978-1980. This prediction is based on the recent past in which major mutations occurred in 1946, 1957, and 1968. This series of conferences will have the objective of developing ideas and directions for research in influenza which will contribute to development of NIAID programs.

Proposed Course of Project: It is anticipated that this contract will be renewed.

Date Contract Initiated: June 30, 1971.

Current Annual Level: $42,000 (estimate)

NATIONAL CENTER FOR DISEASE CONTROL - (Intraagency Agreement) - 71A17

Title: Smallpox Serologies

Contractor's Project Director: James Nakano, M.D.

Project Officer: George Galasso, Ph. D.

Objectives: Perform vaccinia hemagglutination-inhibition (HI) and serum neutralization tests (SNT) on 800-1000 serums.

Major Findings: Contractor is acting as the reference laboratory for the four collaborative smallpox clinical studies. The reference laboratory is responsible for testing 1/4 of the serum samples and correlating results from the other laboratories. Results obtained by the reference laboratory indicate that the four clinical investigators are obtaining similar results with the HI test. Not enough NT results are available to correlate.

Significance to NIAID Programs & Bio-Medical Research: There is currently much concern with the smallpox vaccine due to the infrequent resulting complications. There are some who advocate abolishing compulsory vaccination. This would result in possibly greater problems. A more practical solution would be to get a better vaccination procedure. The Infectious Disease Branch has initiated the collaborative smallpox study of which this contract is a part as an immediate step toward this goal.

Proposed Course of Project: To continue until smallpox collaborative clinical trial has been completed.

Date Contract Initiated: July 1, 1970

Current Annual Level: $ 7,000

NATIONAL DRUG COMPANY - (PH43-69-2072)

Title: Research and development of respiratory vaccines (parainfluenza and adenoviruses)

Contractor's Project Director: Armand N. DeSanctis, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To prepare and safety test zonal purified ten-fold concentrated inactivated parainfluenza type 3 vaccine.

Major Findings: Soluble antigen adenovirus vaccines, types 1, 2, and 5 were prepared under this contract and clinically evaluated under another contract. It was found that the guinea pig ED_{50} correlated very well with antigenicity of the vaccines in adult humans. An ED_{50} of 1:400 in guinea pigs corresponded with 100% seroconversion in humans, while 1:40 corresponded with 75% seroconversion and 1:17 corresponded with 60% seroconversion.

Three lots of monovalent live high passage parainfluenza virus vaccines have been prepared by National Drug Company and clinically evaluated in adult humans. All three lots consisting of types 1, 2 and 3 did not infect adults, and did not cause any adverse reactions in adults. Type 1 has been evaluated in children 6-14 years of age with about 50% infectivity, and no adverse reactions. It is now being evaluated in children 2-6 years of age. Type 3 is being evaluated in children 6-16 years of age and if it is safe in all and infective in some, it will be evaluated in younger children.

Parainfluenza virus type 3 was concentrated and purified by zonal centrifugation. It was then inactivated, one-half buffered with 5% lactose and one-half without buffer. An accelerated stability test revealed that the preparation was very antigenic initially, but that it was not stable. Additional studies of buffers and other parameters are needed.

Significance to NIAID Program and Bio-Medical Research: This contract has provided experimental vaccines which were assessed at various contractor evaluation centers in the program.

Proposed Course of Project: Terminated on February 29, 1972.

Date Contract Initiated: Contract PH43-63-79 was initiated November 29, 1962. The contract number was changed to PH43-69-2072 in May 1969.

Current Annual Level: $5,000 (2 months)

UNIVERSITY OF NEW MEXICO (NIH 71-2202)

Title: Epidemiology of pneumococcal pneumonia among the Navajo Indians

Contractor's Project Director: Robert Oseasohn, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Direct and coordinate epidemiological studies to determine the incidence of pneumococcal disease (pneumonia, otitis media and meningitis) in the Navajo Indian population receiving medical health care at the Gallup PHS Indian Hospital, New Mexico 2) conduct clinical testing of pneumococcal vaccines for the purpose of ascertaining safety and antigenicity in selected volunteers.

Major Findings: This is a collaborative project with the Indian Health Service (Project Director, Dr. George Bock) where a research epidemiological team has been established at the Gallup PHS Indian Hospital to carry out a feasibility study to determine if the population living within the geographic area served by the Gallup Service Unit is an appropriate population group to conduct a vaccine field trial. In an attempt to seek answers to several basic questions regarding population census and whether it is possible to monitor participants in a proposed field trial situation, a field surveillance program has been established to conduct family visitation on a bi-weekly basis. Results from this field surveillance program indicate that the majority of individuals with acute respiratory illness seek the Gallup Hospital for medical care. An annual crude incidence rate for total pneumonia in individuals over the age of 15 years has been estimated at 20 cases/1000. In addition, there appears to be a very low frequency of mortality due to pneumococcal pneumonia in the homes. Results from this feasibility study suggest this subgroup of Navajo Indians would be ideally suited for a vaccine field trial. Clinical trials with monovalent vaccines (types 1, 3 and 8) to determine safety and antigenicity are in progress. Varying doses (12.5, 25, 50 and 100 ug) of each univalent vaccine have been administered to generate antigen extinction data. Serological response is measured by a radioimmunoassay and passive hemagglutination test.

Significance to NIAID Programs and Bio-Medical Research: These studies will establish the firm incidence rate of pneumococcal disease in a well defined population base. This target population with a high frequency of pneumococcal pneumonia has great potential for a large field trial to demonstrate efficacy of a pneumococcal polyvalent vaccine.

Proposed Course of Project: This contract will be reviewed and evaluated before the end of the contract period, May, 1972.

Date Contract Initiated: June, 1971

Current Annual Level: $90,000 (18 months)

NEW YORK MEDICAL COLLEGE - (PH43-68-1266)

Title: Therapeutic Use of Inducers of Interferon for Viral Infections of Animal and Human Eyes

Contractor's Project Director: Miles A. Galin, M.D.

Project Officer: Samuel Baron, M.D.

Objectives: This is a study to evaluate the value of polyinosinic·polycytidylic acid, an inducer of interferon, in the treatment of viral diseases of the human eye, primarily herpetic keratoconjunctivitis.

Major Findings: This first phase of the project consisted of the evaluation of ocular and systemic toxicity of Poly (In·Cn) in six blind volunteers treated as in-hospital patients with a total dose of 4,900 micrograms by topical administration. There were no ocular toxic reactions or systemic toxicity as demonstrated by a series of blood studies taken prior to, during and after therapy.

Six patients who had been subject to recurrent herpetic keratoconjunctivitis were evaluated for toxicity of In·Cn when they were in a non-infected state. All ocular and laboratory tests done in the blind controls were repeated in these patients. No toxicity was found.

The patients in the double-blind study of In·Cn or IDU (A or B) were divided into three diagnostic groups (I) acute herpetic keratoconjunctivitis, treated with In·Cn or IDU; (II) chronic recurrent herpetic keratoconjunctivitis with epithelial lesions, treated with In·Cn or IDU; (III) chronic recurrent herpetic keratoconjunctivitis without epithelial lesions, treated with In·Cn or artificial tears (Tearisol) (C or D). Patients in Group I responded well. Patients in Group II became worse. Most patients in Group III had no recurrences. It would appear that treatment of chronic keratoconjunctivitis is ineffective. The role of IDU and In·Cn in the treatment of acute cases of herpetic keratoconjunctivitis still remains to be determined because of the relatively few numbers of acute patients treated. Therefore, there is still not sufficient data to fully evaluate the efficacy of In·Cn.

Significance to NIAID Programs and Bio-Medical Research: Polyinosinic·polycytidylic acid is an effective inducer of interferon. This topical treatment, if effective, would solve an important medical disease problem which is common and very painful to the patient. The current therapy, although useful, needs improvement.

Proposed Course of Project: It is anticipated that this contract, if it can be supplemented by other contractors doing similar studies, should be completed with FY 72 funding and terminated in June 1973.

Date Contract Initiated: June 20, 1968

Current Annual Level: $87,000 (Estimate)

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-69-2222)

Title: Longitudinal Evaluation of Vaccines for the Control of Rubella

Contractor's Project Directors: Saul Krugman, M.D. and Louis Z. Cooper, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To conduct: 1) longitudinal studies of vaccine efficacy and safety, 2) studies of unattenuated (natural) rubella and congenital rubella and 3) to develop improved techniques for characterizing the immunology of rubella infection.

Major Findings: Longitudinal study of serum antibody in children living in a rubella-free environment showed progressive declines in rubella HI antibody titers following rubella and rubella vaccination. In contrast, a similar rate of titer decline has not been observed among children and adults living in the community. Approximately 20% of children with congenital rubella have rubella HI titers of <1:8 by age 5 to 6 years. These children do not have significant boosts in titer when given rubella vaccine.

Prenatal antibody screening followed by postpartum immunization was found to be safe and practical. Contraception, side reactions and spread of vaccine to infants were not problems. Combined live virus vaccines (measles, mumps, rubella or measles, rubella) gave excellent seroconversion rates.

Administration of antirubella human immunoglobulin shortly after rubella virus challenge modified the clinical course and viral immunology of the infection. Viremia could no longer be detected, pharyngeal shedding and the rubella specific IgM response were decreased.

Techniques for community surveillance of rubella in pregnancy and congenital rubella were improved and additional data obtained. Seventy pregnancies complicated by rubella vaccination are being followed.

Improved reliability resulted from modification of techniques for determining total rubella HI antibody and rubella specific IgM antibody.

Significance to NIAID Program and Bio-Medical Research: This contract will answer a number of questions concerning immunity following natural infection and immunity following vaccination. The widespread utilization of rubella vaccines makes it mandatory to assure the continued protective efficacy and safety of these vaccines.

Proposed Course of Project: Surveillance studies will be continued until June, 1974.

Date Contract Initiated: June, 1969.

Current Annual Level: $113,000. (Funds obligated during FY 1969.)

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-70-2131)

Title: The Evaluation of Topical, Intralesional and Systemic Interferon and Interferon Inducers in the Prevention and Treatment of the Fibroma Virus-Induced Skin Lesion in Rabbits

Contractor's Project Director: Alvin E. Friedman-Kien, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To provide an animal-model system which closely approximates a disease entity in man for the purpose of examining antivirals as they become available to assess their potential value. This particular system is for viral diseases of the skin.

Major Findings: This model provides us with lesions that can be easily observed and used for the systemic, topical and intralesional administration of antiviral agents. During the preceding contract year it was shown that a single systemic 90,000-unit dose of rabbit kidney cell interferon given on the day of viral infection or a single 40,000-unit dose given on the fourth day after infection provided excellent suppression of the virus-induced lesion. In a collaborative study involving isoprinosine, it was determined that the drug was somewhat effective in the prevention of fibroma lesions, although it was ineffective against vaccinia and all the other systems studied in the animal-model contracts. Tilorone was found to be ineffective in rabbits. This group has also developed a herpes cutaneous lesion model in the hairless mouse which is effective both in demonstrating cutaneous lesions and subsequently, encephalitis.

Significance to NIAID Programs and Bio-Medical Research: Having firmly established baseline data on the effects of interferon and interferon inducers in these animal models, the program will be better able to directly test new antivirals as they are developed in the selected systems. This will afford the program with a direct means of comparison in determining the relative merits of the various substances and their potential for clinical use.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974.

Date Contract Initiated: May 25, 1970

Current Annual Level: $53,000

NEW YORK UNIVERSITY MEDICAL CENTER - (NIH-70-2169)

Title: Methods for the Production of Human Interferon

Contractor's Project Director: Jan T. Vilcek, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Develop a technique for a more efficient, safe and economically feasible production of human interferon. A large number of commercially available cells will be investigated for this purpose. Clones will be obtained from the more promising cells and the enhancing effect of anti-metabolites studied. The contractor will concentrate on manipulation of inducers and inducing factors in order to increase the yield of the interferon to practical levels.

Major Findings: A great deal of information has been generated in fulfilling the objectives of the workscope through the exploration of enhancers, super-induction, priming, and regulation of variables in the tissue culture medium. The contractor has concentrated on foreskin fibroblast cells and human embryo kidney cells in his attempts at superinduction. Although moderate success has been achieved, increasing the yield better than five-fold, the high yields obtained with other systems such as the rabbit and mouse systems have not been achieved. Since it is fairly well established that the ten-fold further increase hoped for can be achieved, this contractor will continue with his attempts at superinduction.

Significance to NIAID Programs and Bio-Medical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: May 25, 1970

Current Annual Level: $42,000

UNIVERSITY OF NORTH CAROLINA - (PH-43-67-48)

Title: Longitudinal Studies of Acute Respiratory Disease in Children

Contractor's Project Director: Floyd W. Denny, Jr., M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To develop populations of young children who may be followed Longitudinally for the purposes of observing clinical and epidemiological factors related to acute respiratory disease caused by specific agents; to provide defined populations for the evaluation of methods of prophylaxis, including the use of vaccines, against specific respiratory pathogens.

Major Findings: During the past six years, these investigators have worked closely with the pediatric populations of Chapel Hill, and they have excellent rapport with the community and physicians in charge of giving medical attention to the children of the community. These investigators have done epidemiology surveys of the pediatric population at Chapel Hill, and have found that the most serious life-threatening disease among infants less than 4 months of age is respiratory disease; their studies show that RS virus is the most important cause of these lower respiratory diseases. These studies include an examination of the factors associated with RS disease, such as: cellular immune response, complement depletion in severe RS disease, and the relationship of host factors such as the level of maternal antibodies, breast feeding and oral poliovirus administration on the occurrence of RS virus infection. A few initial vaccine trials have been done with the candidate attenuated RS virus vaccine (ts-1 mutant), so these workers are well aware of the problems of vaccine evaluation in the Chapel Hill community.

Significance to NIAID Program and Bio-Medical Research: It is necessary to have a background of continuous respiratory disease surveillance in order to evaluate methods of respiratory disease prophylaxis.

Proposed Course of Project: The contract was terminated on March 31, 1972.

Date Contract Initiated: November 14, 1966.

Current Annual Level: $20,000 (3 months)

THE UNIVERSITY OF NORTH CAROLINA AT CHAPEL HILL (NIH 72-2505)

Title: Clinical evaluation of respiratory vaccine in children and infants

Contractor's Project Director: Floyd W. Denny, Jr., M.D.

Project Officers: Robert M. Chanock, M.D. and June K. Dunnick, Ph. D.

Objectives: The contractor will conduct clinical evaluation of live virus vaccines with a major emphasis on the evaluation of live respiratory syncytial virus (RS) vaccine in a closed population of infants to ascertain infectivity, lack of virulence and production of antibody; and in semi-closed or carefully monitored open populations of children and infants to obtain additional information on infectivity, lack of virulence, production of antibody and transmissibility.

If the need should arise, and at the direction of the Project Officer, the contractor will conduct clinical studies to evaluate reactivity and antigenicity of candidate bacterial polysaccharide vaccines, specifically *Hemophilus influenzae* type b polyribose phosphate vaccine.

Major Findings: Since this is a new contract, there are no findings to report.

Significance to NIAID Program and Bio-Medical Research: This contract is part of the schedule of the respiratory virus vaccine development program; it is necessary to do careful clinical trials on vaccines that are likely to be effective in reducing respiratory illnesses.

Proposed Course of Project: The contract has been funded through March 31, 1973

Date Contract Initiated: April 1, 1972

Current Annual Level: $39,961

OHIO STATE UNIVERSITY (PH43-67-78)

Title: Influence of cultural condition and concentration procedures on the antigenicity of Mycoplasma pneumoniae vaccines

Contractor's Project Director: Norman Somerson, Ph. D.

Project Officer: Robert Purcell, M.D.

Objectives: 1) Produce 20,000 ml of an experimental M. pneumoniae vaccine, using strain C18 2) develop a process suitable for large-scale commercial production of vaccine.

Major Findings: The source of bacterial contamination (Vibrio percolans) which had plagued large-scale vaccine production at HRC has been eliminated at the Ohio State facility. A variety of improved steps have been devised which enhance the antigenic potency as measured in the hamster animal model. More reliable results by the MI test have been achieved by substituting a 0.1% glucose rinse for the PSS wash. The 0.1% glucose wash minimizes the disruption of cells and promotes a more accurate assay of viable organisms. An improved test for purity has been devised. This includes a neutralization test with specific antibody whereby a test is made for breakthrough. The active ingredient of the bovine serum fraction has been characterized as a lipoprotein material with a molecular weight of approximately 200,000. Growth promoting activity is directly related to the concentration of the material. It appears unstable when refrigerated, but stable when bovine serum fraction is frozen at -20°C. Studies are in progress to assess stablility of the vaccine when stored up to one year under five conditions: 1) room temperature 2) 4°C 3) -20°C 4) -70°C and 5) lyophilized. A new scheme for potency testing has been initiated. Each sub-lot will be assayed for potency before combining sub-lots into a batch. Thus, only high-potency sub-lots will be pooled into final batches. Plans are being made to clinically evaluate the candidate vaccine for safety and antigenicity starting in July, 1972.

Significance to NIAID Programs and Bio-Medical Research: There is little known about the antigenic composition of Mycoplasma preparations obtained from organisms grown under a wide variety of conditions. Such studies are an important prerequisite for production of a potent Mycoplasma pneumoniae vaccine. The new improved methodology for vaccine production would make a M. pneumoniae vaccine commercially feasible.

Proposed Course of Project: This contract will be revised and evaluated prior to its anniversary date.

Date Contract Initiated: December, 1966

Current Annual Level: $124,250

MEDICAL COLLEGE OF PENNSYLVANIA - (NIH-70-2126)

Title: Production of Anti-Interferon Sera and Development of Immunoassays

Contractor's Project Director: Kurt Paucker, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Apply the techniques developed with mouse interferon to produce anti-human interferon sera. This material will be sufficiently specific to inhibit the antiviral effect of interferon.

Major Findings: The contractor has successfully produced high-titered anti-mouse interferon serum in rabbits. Nonspecific antibodies were absorbed from the antisera, and the data show that the interferon neutralizing antibody was specific for interferon with no detectable antibodies to normal mouse constituents. A limited amount of this anti-mouse interferon antisera has now been made available for other investigators.

Significance to NIAID Programs and Bio-Medical Research: Specific antisera to interferon can be used to develop an immunoassay and, more important, in studies on mechanisms of interferon induction. When an inhibitory reaction is detected, the antisera can be used to determine whether the reaction is due to interferon or some other inhibitory effect.

Proposed Course of Project: To be reviewed and the results evaluated and considered for renewal at the end of the contract year.

Date Contract Initiated: June 1, 1970

Current Annual Level: $40,000

THE MEDICAL COLLEGE OF PENNSYLVANIA - (NIH-70-2172)

Title: Production of Interferon in Human Cell Lines

Contractor's Project Director: Kurt Paucker, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Develop a technique for more efficient, safe and economically feasible production of human interferon. This contractor will study various strains of diploid and heteroploid cells for the production of interferon. He will attempt to enhance production by priming cells and using inhibitors of protein and RNA synthesis. The techniques devised will be of such a nature that the ultimate product may be used in man.

Major Findings: Five stationary human cell lines (diploid and heteroploid) were studied for their interferon-producing potential using nine viral inducers and poly I·poly C. Newcastle disease virus and Sendai virus gave best production of interferon. Ultraviolet irradiation of Newcastle disease virus resulted in up to 8-fold increases in inferferon yields. Omission of the serum from the collection medium did not impair interferon production. Variation of temperature and substances which enhance uptake of large molecules did not increase interferon production. Priming of interferon production by pretreatment of two cell lines with either virus, poly I·poly C or human interferon did not increase yields. Two lymphoblastoid lines of normal human cells in spinner culture were studied for their interferon-producing potential. Heteroploid cell lines did not yield more interferon than did diploid cell lines.

Significance to NIAID Programs and Bio-Medical Research: All the interferon inducers currently available are toxic; therefore, it is possible that the only effective use of interferon may be the direct application of interferon produced externally. If this is the case, a more efficient and economically feasible means of producing interferon must be found.

Proposed Course of Project: The work outlined for this project will be completed within FY 72 and the contract terminated.

Date Contract Initiated: June 1, 1970

Current Annual Level: (prior year support)

TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA (PH43-67-681)

Title: Epidemiology of pneumococcal disease in preparation for field trials with pneumococcal vaccine

Contractor's Project Director: Robert Austrian, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Continue to determine, on the basis of wide geographic sampling, pneumococcal serotypes most often responsible for invasive infection in adults and in children 2) to ascertain the frequency of bacteremic and non-bacteremic pneumonia and of otitis media caused by specific pneumococcal serotypes in defined populations 3) to develop further information concerning the immunological response of man to pneumococcal infection and 4) to test monovalent and polyvalent vaccines of pneumococcal capsular polysaccharides in human volunteers for safety and antigenicity.

Major Findings: Significant data have been accumulated on the prevalence of pneumococcal disease in high-risk populations throughout the world. Particular population groups have been defined (Navajo Indians of Arizona, subscribers of the Kaiser Permanente Group of San Francisco and the Dorothea Dix Hospital, Raleigh, North Carolina) where there is an exceedingly high prevalence of natural disease and where studies could be structured to perform intense epidemiologic surveillance in conjunction with field trials to show efficacy of new experimentally developed pneumococcal polyvalent vaccines. Studies on acute otitis media in children reveal that 64.8% of the strains of pneumococci recovered from the middle ear exudate are types 1, 3, 6, 14, 19 and 23. Preliminary studies indicate that the human antibody response to natural infection and vaccination with capsular polysaccharide includes both 19S and 7S antibodies. It is clearly evident that the antibody most active in the passive HA test is in the 19S fraction, although occasionally some hemagglutinating activity is demonstrable in the 7S fraction. Studies are in progress to determine the relative protective effects of the several antibody fractions against homotypic infection in mice. Phase I testing of monovalent vaccines 1, 3, 4, 7 and 12 and a candidate hexavalent vaccine containing these same serotypes has been performed. No serious untoward reactions have been observed to follow the immunization of more than 700 volunteers. Completion of clinical trials with the following monovalent pneumococcal vaccines (types 6, 9, 14, 18, 19 and 23) for safety and antigenicity are planned for the last quarter of FY '72.

Further refinement of the radioimmunoassay (RIA) procedure have been developed. More highly purified C^{14}-labeled capsular antigens of types 1, 4 and 7 have been obtained by precipitating the crude antigens with homologous capsular antibody, disassociating the complexes at pH_2 with citrate buffer and recovery of the disassociated antigen by column filtration. It is anticipated that more high specific antigen will be prepared as reference antigen for other assay systems.

Significance to NIAID Programs and Bio-Medical Research: Epidemiological analysis of clinical and immunological data will aid in the interpretation of vaccine field trials to demonstrate efficacy of newly developed pneumococcal vaccines.

Proposed Course of Project: The contract will be reviewed and evaluated for renewal at the end of the contract year, May, 1972.

Date Contract Initiated: February, 1967

Current Annual Level: $327,000 (estimate)

UNIVERSITY OF PITTSBURGH - (NIH-70-2128)

Title: Development of Better Rabbit Interferon Standards and Assay Systems

Contractor's Project Director: Monto Ho, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Study the stability and potency of the wet frozen ampouled rabbit interferon standard and the possibility of obtaining a lyophilized standard.

Major Findings: This laboratory was designated as the producer of the international research reference rabbit interferon standard. This was accomplished in studies on the stability, both wet frozen and lyophilized, undertaken. The wet frozen standard has been tested in five different laboratories and proven reliable. Studies are currently under way on the stability of the lyophilized rabbit interferon. The contractor continues to monitor the rabbit interferon standard for stability.

Significance to NIAID Programs and Bio-Medical Research: The NIAID is currently supplying this rabbit interferon as the international standard, therefore, careful control of its stability must be maintained. Further studies on lyophilization are needed to determine whether the material could be dispersed in this form which would be more desirable.

Proposed Course of Project: This contract was terminated on June 9, 1972.

Date Contract Initiated: June 10, 1970

Current Annual Level: (prior year support)

UNIVERSITY OF ROCHESTER - (NIH-72-2503)

Title: Conduct clinical evaluation of respiratory viral and bacterial vaccines in children and infants.

Contractor's Project Director: R. Gordon Douglas, Jr., M.D.

Project Officer: Robert Chanock, M.D.

Objectives: Clinical evaluation of live attenuated virus vaccines (RSV, parainfluenza and adenovirus) and of inactivated mycoplasma pneumoniae vaccine. The major effort to be placed on testing respiratory syncytial virus vaccine in closed populations of infants to ascertain infectivity, lack of virulence and production of antibody.

Clinical studies to evaluate reactivity and antigenicity of candidate bacterial polysaccharides with major emphasis on Hemophilus influenzae type b polyribose phosphate (PRP) vaccine in children.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: The data obtained from these studies will help to establish the safety and antigenicity on new prototype respiratory viral and mycoplasma pneumoniae vaccines being developed by the Infectious Disease Branch. Studies with H. influenzae (PRP) vaccine will aid immensely in developing an optimal immunization schedule for this vaccine in young children and infants.

Proposed Course of the Project: To be reviewed and evaluated for renewal at the end of the contract year.

Date Contract Initiated: May 1972

Current Annual Level: $120,000

THE ROCKEFELLER UNIVERSITY (NIH 71-2502)

Title: Radioimmunoassay for the quantitative assay of human anti-meningococcal antibodies.

Contractor's Project Director: Emil Gotschlich, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Establish a reference serological laboratory to measure specific antibody to groups A, B and C meningococcal polysaccharide 2) develop a standardized radioimmunoassay (RIA) including reference antigen and sera, to measure serum antibody response of children and infants to varying doses of meningococcal groups A, B and C polysaccharide vaccines 3) correlate quantitative data of the antibody response as measured by the RIA with other serological procedures (HA, fluorescent antibody test, bactericidal assay and the opsonic test) that are employed to assess similar immune response.

Major Findings: During the first year of this project, the contractor has developed an RIA procedure including radio labeled reference antigen and reference antiserum to standardize the assay procedure for measuring the response of children to group A meningococcal polysaccharide. The second major effort has been to develop a radioimmunoassay procedure for the group C and the group B meningococcal polysaccharide as well. Considerable progress has been made in this direction.

Preliminary studies have shown that children respond immunologically to groups C and A meningococcal polysaccharide and the vaccines are not toxic up to at least 250 mcg of antigen. The hemagglutination technique which has been adequate for measuring antibody elicited in adults is not sufficiently sensitive in children; the RIA procedure has been demonstrated to provide this needed sensitivity. In a pilot study involving 48 children between the ages of 7 months and 9 1/2 years, the geometric mean antibody concentration (ug antibody protein/ml) was 10.3 ug for the group receiving the A polysaccharide and 14.5 ug for the group receiving the C polysaccharide vaccine. Detectable levels of specific antibody were found in the sera of children 10 to 15 months after vaccination with group A or group C antigen. Studies are in progress to compare and correlate the RIA immune response with parallel values determined by the bactericidal assay and the opsonic test.

Significance to NIAID Programs and Bio-Medical Research: There is a need to develop a highly sensitive and quantitative serological procedure to measure low levels of antibody in infants and children recovering from meningococcal meningitis and those who have been actively immunized with purified meningopolysaccharides. Combined development of the RIA procedure has the potential of achieving this goal. The services of a reference serological laboratory is essential for a comprehensive assessment of the antigenic and protective capabilities of meningococcal vaccine for children and infants.

Proposed Course of Project: This contract will be reviewed and evaluated before the anniversary date, May, 1972.

Date Contract Initiated: June, 1971

Current Annual Level: $26,600

RUTGERS UNIVERSITY - (NIH-72-2504)

Title: Development of Attenuated Mutants of Respiratory Syncytial Virus and Parainfluenza viruses as Potential Vaccine Strains

Contractor's Project Director: Robert W. Simpson, Ph.D.

Project Officer: Robert M. Chanock, M.D.

Objectives: By the use of mutagenic agents, and/or viral recombination, and/or other methods, prepare and evaluate new viral strains of respiratory syncytial virus and parainfluenza viruses, types 3, 1 and 2 with characteristics suitable for use as potential vaccine strains. The attenuated viruses should be infective, less virulent that the parent virus, and induce local and/or serum neutralizing antibody in appropriate test animals, e.g. hamsters.

Major Findings: Because this is a new contract, no results are available.

Significance to NIAID Program and Bio-Medical Research: Respiratory syncytial virus (RSV) and parainfluenza type 3 virus cause a highly significant amount of sever illness in young children. A safe, effective vaccine to these viruses is of the highest priority.

Proposed Course of Project: Renewal is anticipated.

Date Contract Initiated: June 1, 1972

Current Annual Level: $80,000

ST. LOUIS UNIVERSITY SCHOOL OF MEDICINE - (NIH-70-2266)

Title: Smallpox Vaccine Evaluation

Contractor's Project Director: James D. Cherry, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study and compare the available smallpox vaccines by two routes, subcutaneous and percutaneous, in three different dosages with the intent of obtaining information for selecting a better vaccine and/or vaccination procedure so as to reduce the complications resulting from the immunization.

Major Findings: A collaborative study to evaluate the reactivity and immunogenicity of four vaccinia viruses when administered percutaneously for primary vaccination in infants and children is in progress. Six months following the primary vaccination a percutaneous challenge with the present licensed vaccine is administered. Serum antibody response are being measured by the hemagglutination inhibition and the neutralization tests. Very little of the post-challenge antibody data is available. Since this is a blind random study, a statistical analysis of the results will not be available until the end of the study. However, it appears that a primary subcutaneous immunization with a low dose of vaccinia virus results in lowered morbidity when the current licensed vaccine is administered percutaneously.

A total of 750 infants have been immunized. About 10% of those receiving the vaccines by the subcutaneous route exhibited redness at the site of immunization and/or arm enlargement (by measurement).

Significance to NIAID Programs & Bio-Medical Research: The recommendations of the Public Health Service and the Pediatric Society are to discontinue routine smallpox immunizations. Recent outbreaks of smallpox may influence these recommendations. It is hoped that the present collaborative study will result in information that can be used to determine a better vaccination procedure -- whether or not it is used routinely.

Proposed Course of Project: Contract was awarded through December 1972.

Date Contract Initiated: June 12, 1970

Current Annual Level: $114,700 (18 months FY '71 funds)

SMITH, KLINE AND FRENCH LABORATORIES, PHILADELPHIA, PA.

Title: Partial Purification, Safety Testing, and Preparation of Concentrated Human Leucocyte Interferon for Clinical Trial

Contractor's Project Director: Richard F. Haff, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: The state of the art has now advanced to the point that exogenous interferon can be tested for efficacy in a clinical trial. The purpose of this contract is to process interferon (concentrate, partially purify, safety test and ampule) for a clinical trial on varicella-zoster in the immunologically suppressed patient.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: One of the initial aims of the Antiviral Substances Program was and continues to be to determine whether exogenous interferon can be used efficaciously in man. This contract is an integral part of an attempt to test this material.

Proposed Course of Project: It is anticipated that the contract will be completed within one year.

Date Contract Initiated: June 1972

Current Annual Level: $36,000

STANFORD UNIVERSITY - (NIH-71-2201)

Title: Methods of Increasing Resistance of Polyinosinic·Polycytidylic Acid to Human Serum Hydrolytic Enzymes

Contractor's Project Director: Thomas Merigan, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: To study chemical modifications of the chemical inducer polyinosinic·polycytidylic acid in order to stabilize it against human serum hydrolytic enzymes. Ultimate goal is to obtain an effective, less toxic inducer for use in man.

Major Findings: A series of new chemical inducers which are modifications of poly I·poly C have been produced and tested in selected models by the contractor. This study is currently at the half-way point. The results to date do not look very promising in terms of obtaining a more efficient inducer through chemical modification of poly I·poly C.

Significance to NIAID Programs and Bio-Medical Research: Interferon remains the most promising antiviral currently available for study toward feasibility for use in man. Two methods of administration are available: exogenous, prepared in vitro, and endogenous, through use of inducers. The inducers available are somewhat toxic, including the most promising, poly I·poly C. This contract is an attempt to stabilize the compound so that the dosage can be reduced without affecting or increasing its inducing abilities.

Proposed Course of Project: This contract has been extended for an additional year with no additional funds. It is expected that the work proposed should be completed and the contract terminated in June of 1973.

Date Contract Initiated: June 1971

Current Annual Level: $59,700 (FY '71)

TULANE UNIVERSITY - (PH43-63-75)

Title: Vaccine Studies in Various Population Groups

Contractor's Project Director: William Mogabgab, M.D.

Project Officer: Albert Kapikian, M.D.

Objectives: 1) To maintain respiratory disease surveillance in dependent children at Keesler Air Force Base with respect to incidence, clinical features, and specific etiology. 2) To determine protective effects of respiratory disease vaccines shown to be needed by the results of surveillance. 3) To characterize new agents including variants of known agents. Bacteria, mycoplasma and viruses will be included.

Major Findings: Three lots of inactivated M pneumoniae vaccine were tested for safety and antigenicity in young adult males. The vaccines were antigenic but produced tenderness and erythema at the site of injection.

Adenovirus type 5 soluble antigen vaccine, DNA-free, was evaluated for antigenicity and safety in nine adults. One experienced anorexia, nausia and vomiting which was temporarily related to vaccine administration. Another vaccinee experienced wheezing relieved by antihistamines, and swelling of the right periorbital tissue about 45 minutes following vaccination. Safety tests were redone on this vaccine and no explanation for the allergic response was found. The two unexplained systemic reactions seen following vaccine administration made further clinical evaluation of this vaccine unwarranted.

Significance to NIAID Program and Bio-Medical Research: Provides populations suitable for vaccine studies.

Proposed Course of Project: The contract was terminated on March 31, 1972.

Date Contract Initiated: December 14, 1962.

Current Annual Level: $15,032 (3 months)

TULANE UNIVERSITY - (NIH-70-2124)

Title: Factors Influencing the Assay of Interferon

Contractor's Project Director: Jules V. Hallum, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: In order to expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay. Toward this end this contract will examine a variety of conditions such as differences due to volume of interferon used, cell concentration in test system, suspension versus monolayer cultures, type of endpoint and relationship of time between infection and harvest of challenge virus in the assay.

Major Findings: The effect of variables, such as volume, cell types and cell concentration, on the sensitivity of interferon assays were studied. Comparison of the relative sensitivity of different types of endpoints indicates that yield reduction is the most sensitive, plaque reduction the next most sensitive and CPE reduction the least sensitive. This contractor has contributed much needed information on the variables of the assay system, which contributes to a better understanding as to the problems involved in obtaining consistent results.

Significance to NIAID Programs and Bio-Medical Research: The methods currently in use to assay interferon are tedious, time-consuming, and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: This contract was terminated on June 21, 1972.

Date Contract Initiated: June 22, 1970

Current Annual Level: (prior year support)

TULANE UNIVERSITY, DELTA REGIONAL PRIMATE RESEARCH CENTER (NIH-71-2197)

Title: Studies of response of sero-negative chimpanzees following exposure to hepatitis B antigen (HB Ag)

Contractor's Project Director: Dr. Peter Gerone

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: To furnish healthy sero-negative chimpanzees and to provide for all necessary care of these animals, including veterinary care; these chimpanzees will be housed under isolated conditions for the duration of the experiments. The contractor will obtain blood samples, biopsy specimens, make clinical observations and furnish laboratory services.

Major Findings: The contractor has identified at least twelve sero-negative chimpanzees (i.e. no HB Ag or HB Ab) suitable for the proposed experiments. However, experimentation has not been started as isolated housing conditions have not been provided. Furthermore, recent work in the area of hepatitis animal model research indicates that the rhesus monkey will prove to be a more practical system for the study of hepatitis B than the chimpanzee.

Significance to NIAID Program and Bio-Medical Research: The chimpanzee has recently been infected with hepatitis B by several workers. Dr. Barker, DBS, has demonstrated clinical or subclinical hepatitis in two of six chimpanzees inoculated with potentially infectious material. Thus, the chimpanzee is one of the few animals that has been shown to get hepatitis B infection, and this information could eventually be used to test prospective hepatitis B vaccines. However, since not all chimpanzees became ill, a study of this nature would require experimentation with more than four animals. In view of the fact that the rhesus monkey is a more practical animal model system, the NIAID plans to support primarily the rhesus monkey work and de-emphasize the chimpanzee work.

Proposed Course of Project: Terminate after the contract year is up.

Date Contract Initiated: June, 1971

Current Annual Level: $48,565 (18 month period)

UNIVERSITY OF UTAH - (NIH-70-2133)

Title: Antiviral Substances and Animal Systems

Contractor's Project Director: Lowell A. Glasgow, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: Provide an animal-model system for examining antivirals against virus diseases similar to those in man. Having provided baseline data concerning interferon and interferon inducers, this study will be available for comparison of new antivirals as they are developed.

Major Findings: The therapeutic efficacy of an oral (Tilorone) and a systemic (poly I·poly C) interferon inducer was determined in mice infected with encephalomyocarditis (EMC) virus. Repeated doses of poly I·poly C were able to give protection against EMC virus in mice, provided the initial dose was early enough. An important condition in obtaining delay in death time was a delay or prevention in onset of viremia. However, even when viremia was abolished, death still occurred in 40% of the animals. It may be that poly I·poly C delays virus growth, but on cessation of treatment the virus growth reensues. It may be that such treated animals do not develop neutralizing antibodies. In model infections of EMC or herpes virus hominis, type 2, isoprinosine had no effect on the course of the disease. Tilorone continues to be effective in mice. This contractor will continue his studies and investigate the adjuvant effectiveness of COAM. He will also look into the effectiveness of IUDR and Ara-A and its effectiveness on the fatality of the mouse encephalitis.

Significance to NIAID Programs and Bio-Medical Research: Having firmly established these animal models, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974.

Date Contract Initiated: June 30, 1970

Current Annual Level: $45,500

UNIVERSITY OF UTAH - (NIH-70-2175)

Title: Study of Interferon and Interferon Inducers in the Prevention of Rabies in Animals

Contractor's Project Director: Burton Janis, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: This contract has a two-fold purpose, the first being to establish an animal-model of the disease which closely mimics a disease state in man in which potential new antivirals can be tested and secondly to serve as a direct model in anticipation of clinical studies on the treatment of rabies where vaccine and interferon would be used simultaneously.

Major Findings: The effects of poly I·poly C combined with exogenous interferon used as an enhancer for interferon production, the protective effects of exogenous interferon and interferon inducers when used in combination with anti-rabies vaccine, and similar experiments on the effect of varying doses of vaccine and poly I·poly C on the prevention of rabies in mice and rabbits have been initiated. The results are currently being tabulated. The contractor will continue his work to determine optimal antiviral effects of the agents being studied to determine the limits of their effectiveness with respect to their time of administration after virus challenge and determine the minimal effective dose of the antiviral, the best sites of administration, the effect of enhancers of the antivirals, and the effects the antivirals may have on the outcome of the disease when used in combination with present day anti-rabies vaccine and antisera treatment.

Significance to NIAID Programs and Bio-Medical Research: Having firmly established these animal models, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances. This contract also serves as a preliminary study toward the adoption of a new method of treatment for rabies in man.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at end of contract year.

Date Contract Initiated: June 17, 1970

Current Annual Level: $39,000

UNIVERSITY OF UTAH - (NIH-71-2200)

Title: Mechanisms of Hyporeactivity to Induction of Interferon

Contractor's Project Directors: Lowell Glasgow, M.D.
Douglas W. Hill, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: One of the major obstacles in clinical studies with interferon inducers is the hyporeactive period. After initial induction of the antiviral substance, a period of 6-7 days is required before more interferon can be induced using the same inducer. The objective of this contract is to devise a methodology to overcome this hyporesponsiveness.

Major Findings: The project consists of the study of natural and synthetic interferon inducers in such a way as to lead to their utilization in sequence without development of an interferon hyporesponsive state. Studies have been undertaken to determine the kinetics of interferon production in mice inoculated by various routes with the interferon inducers poly I·poly C, NDV, and Tilorone. A number of variables have been studied: effects of dose, route of administration, combination of inducers, and their times of inoculation. There is some indication that a combination and alternating of inducers may overcome hyporeactivity, but additional studies are needed.

Significance to NIAID Programs and Bio-Medical Research: If interferon proves to be effective as an antiviral for prophylactic and/or therapeutic treatment of viral diseases, it will be important to be able to overcome the hyporesponsive period.

Proposed Course of Project: To be reviewed, evaluated and considered for renewal at the end of the contract year if there is sufficient indication that the hyporesponsive period can be overcome.

Date Contract Initiated: June 1971

Current Annual Level: $33,000

VANDERBILT UNIVERSITY (NIH 72-2507)

Title: Clinical evaluation of respiratory vaccines in children and infants.

Contractor's Project Director: William F. Fleet, M.D.

Project Officers: Robert M. Chanock, M.D. and June K. Dunnick, Ph. D.

Objectives: The contractor will conduct clinical evaluation of live virus vaccines (respiratory syncytial virus, parainfluenza viruses and adenoviruses) and of inactivated Mycoplasma pneumoniae vaccine. The major emphasis is on the evaluation of live respiratory syncytial virus vaccine in closed populations of infants to ascertain infectivity, lack of virulence and production of antibody, and in semi-closed or carefully monitored open populations of children and infants to obtain additional information of infectivity, lack of virulence, production of antibody and transmissibility.

Major Findings: Since this is a new contract, there are no findings to report.

Significance to NIAID Program and Bio-Medical Research: This contract is part of the schedule of the respiratory virus vaccine development program; it is necessary to do careful clinical trials on vaccines that are likely to be effective in reducing respiratory illnesses.

Proposed Course of Project: The contract is for a period of one year; through March 31, 1972.

Date Contract Initiated: April 1, 1972

Current Annual Level: $129,998

UNIVERSITY OF VERMONT -(PH43-69-91)

Title: Immunogenic and Antigenic Studies of Respiratory Syncytial Virus: Evaluation of Rubella, Respiratory Syncytial Virus and Other Vaccines

Contractor's Project Director: Ben Forsyth, M.D.

Project Officer: Daniel I. Mullally, M.D.

Objectives: To complete the immunological studies on RS virus; to evaluate RS, adenovirus, influenza and other vaccines in selected populations.

Major Findings: RS virus vaccine ts 1 mutant was administered intranasally to children aged 5-7 years. No untoward effects were seen. These children, who had pre-existing RS serum antibody responded with a significant rise in IgA nasal antibody.

A group of chronic bronchitis patients received various types of pneumococcal polysaccharide vaccine prepared by Eli Lilly Co. The vaccines were well-tolerated. It was hoped that administration of a polysaccharide would result in the elimination of carriage of that pneumococcal type, but most carriers remained carriers. Surveillance of this group continues.

Significance to NIAID Program and Bio-Medical Research: Provides important populations for clinical testing of vaccines.

Proposed Course of Project: The contract was terminated on March 31, 1972.

Date Contract Initiated: Contracts PH43-67-76 and PH43-67-77 were initiated in December, 1966, and combined; and the number changed to PH43-69-91 in January 1969.

Current Annual Level: $17,000 (3 months)

VETERANS ADMINISTRATION HOSPITAL, MOUNTAIN HOME, TENNESSEE
Interagency Agreement

Title: Determine epidemiological characteristics of patients with pneumonia

Contractor's Project Director: Irvin S. Perry, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Determine the prevalence of pneumococcal serotypes responsible for pneumococcal pneumonia by appropriate bacteriological examinations of sputum, blood culture, and tracheal secretions. 2) Based on appropriate clinical evaluations and bacteriological surveillance techniques, determine the incidence rate of pneumococcal pneumonia in this population. 3) Conduct preliminary clinical trials with pneumococcal vaccines in volunteers to ascertain safety and antigenicity.

Major Findings: Careful review of chest films and clinical records were used to establish a monthly rate for overall pneumonia. An analysis of the number of pneumococcal isolates from the sputum indicated that approximately 40% of the pneumonia cases were due to pneumococci. Extrapolated to an annual basis, the prevalence of pneumococcal pneumonia is estimated to be 70-75 cases or 20 cases per 1000 individuals. While there is an exceedingly high rate of pneumococcal pneumonia in this population, the stability patterns of men living in the domiciliary units would preclude a successful clinical trial to demonstrate efficacy of the experimental polyvalent vaccine.

Phase I evaluation of a candidate hexavalent pneumococcal vaccine in approximately 20 volunteers is in progress. Final post-immune serum from vaccinees will be taken after 6 months, and type specific antibody levels determined by the radioimmuno assay and passive hemagglutination tests.

Significance to NIAID Programs and Bio-Medical Research: This is one of several epidemiological studies sponsored by the Infectious Disease Branch for developing surveillance in high risk populations in preparation for a vaccine trial.

Proposed Course of Project: The epidemiological pattern of pneumonia in this domiciliary center has been established. This project will be phased out early in FY 73.

Date Contract Initiated: July 1, 1972

Current Annual Level: $12,300

VETERAN'S ADMINISTRATION HOSPITAL, WASHINGTON, D. C.

Title: Efficacy of hyperimmune anti-HB Ab gamma globulin for the prevention or modification of parenterally acquired viral B hepatitis.

Project Directors: H. J. Zimmerman, M.D. and L. B. Seeff, M.D.

Project Officers: Robert H. Purcell, M.D. and June K. Dunnick, Ph. D.

Objectives: The Veteran's Administration Hospital, under the direction of Drs. Zimmerman and Seeff, will conduct a double-blind study administering (IM) either normal gamma globulin or high-titered anti-HB Ag gamma globulin to determine if these preparations lower the incidence of hepatitis B infection in persons experiencing accidental parenteral exposure to hepatitis B. All persons admitted to the study will have no past history of hepatitis, and no past history of positive tests for either HB Ag or HB Ab. Dr. Zimmerman and Dr. Seeff have had excellent experience in planning and executing efficacy trials of gamma globulin for the prevention of hepatitis. Since 1968, the Veteran's Administration Hospital has participated in a VA Cooperative Study of Hepatitis Prevention and thus, bring to this study an already working network of persons involved with hepatitis studies.

Major Findings: Since this is a new contract, no data are reported.

Significance to NIAID Programs and Bio-Medical Research: The NIAID Collaborative Hepatitis B Program has as its goal, control of hepatitis B infection. Generally, control of hepatitis may be divided into two categories: 1) Environmental control, which includes elimination of the carrier state; obtaining high quality blood and blood products; and testing and elimin
HB Ag or hepatitis agents from blood and blood products 2) and control by immunization - either active or passive immunization.

The NIAID - VA Hospital Cooperative Hepatitis Study is designed to determine if hepatitis B infection can be controlled by passive immunization, and this study is thus an integral part of the overall NIAID Hepatitis Program.

Proposed Course of Project: 2-3 years to complete study.

Date Contract Initiated: May 1972

Current Annual Level: $25,000

UNIVERSITY OF VIRGINIA - (PH43-63-1128)

Title: Respiratory Infections in Adults

Contractor's Project Director: Jack Gwaltney, Jr., M.D.

Project Officer: Albert Kapikian, M.D.

Objectives: 1) To obtain scientific information which will define further the etiology and epidemiology of acute respiratory infections in adults and provide bases for the prevention or control of these infections. 2) To clinically evaluate respiratory vaccines.

Major Findings: The antigenicity of experimental M. pneumoniae vaccines prepared from antigen grown in a medium buffered with N-2 hydroxyethylpiperazine- N^1-2-ethane sulfonic acid (HEPES) was tested in young, adult males. Formalin inactivated antigens from a high passage strain and a low passage strain at various dilutions (12-123 ug N/ml) were injected intramuscularly in 1 ml doses. Antibody responses were tested by the metabolic inhibition (MI) technique. Sixty-five to 86% of the volunteers in all vaccine groups responded with fourfold or greater MI antibody rises, but only nine (39%) of 23 antibody free subjects converted compared to 53 (88%) of 60 of those with pre-existing antibody. A booster vaccination did not increase the number of converters or enhance the geometric mean titers. The antigen concentrations of vaccines with 24 or more ug N/ml appeared to be above the threshold needed for maximal antibody responses in the dose range tested. MI antibody rises could not be detected in sputa and nasal washings obtained from a small group of vaccines.

Significance to NIAID Program and Bio-Medical Research: Provides populations suitable for vaccine studies. Provides epidemiological data to establish whether or not there is a need for a particular respiratory vaccine.

Proposed Course of Project: The contract was terminated on March 31, 1972.

Date Contract Initiated: June 5, 1963

Current Annual Level: $23,300 (3 months)

UNIVERSITY OF VIRGINIA - (NIH-72-2508)

Title: Clinical Evaluation of Influenza Virus and *Mycoplasma Pneumoniae* Vaccines

Contractor's Project Director: Jack Gwaltney, Jr., M.D.

Project Officer: Albert Z. Kapikian, M.D.

Objectives: 1) To clinically evaluate attenuated influenza virus vaccines administered by the intranasal route in appropriate populations to show infectivity, lack of virulence and efficacy. 2) To clinically evaluate inactivated *M. pneumoniae* vaccine for safety and antigenicity in young adults. 3) To clinically evaluate inactivated influenza virus vaccines administered parenterally to show safety, antigenicity and efficacy.

Major Findings: Because this is a new contract, there are no results at this time.

Significance to NIAID Program and Bio-Medical Research: Contractor provides unique populations which have been under surveillance, so that efficacy studies of influenza virus vaccines can be performed.

Proposed Course of Project: Renewal is probable.

Date Contract Initiated: April 1, 1972

Current Annual Level: $71,028

UNIVERSITY OF WASHINGTON (NIH 71-2280)

Title: Vaccine evaluation and epidemiological studies on pneumococcal disease

Contractor's Project Director: J. Thomas Grayston, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Provide information on the incidence of pneumococcal pneumonia and otitis media in a large open population located in and around Seattle, Washington 2) continue to develop a monkey model to study experimental infection and immune response with selected pneumococci (types 1-9, 12, 14, 18, 19 and 23).

Major Findings: An intensive epidemiological surveillance study was established at the Puget Sound Group Health Cooperative Hospital of Seattle. The total pneumonia rate for this open population of 140,000 subscribers is estimated at 10 cases per 1000 subjects.

The proportion of pneumonia associated with M. pneumoniae or respiratory virus were found for the following:

	M. pneumoniae	Viruses	Neither
Children	21%	30%	49%
Adults	15%	18%	67%

In more than half of the pneumonia patients, no etiologic agent was found. Patients with pneumonia, without any evidence of viral or mycoplasmal infection, had a higher rate of asthma, chronic pulmonary conditions and other predisposing diseases than those who had evidence of infection with one of the agents. A high proportion of patients with pneumococci recovered from throat swabs had evidence of infection with M. pneumoniae or viruses. The isolation rate of pneumococci was highest among 40-60 year old individuals.

Challenge studies have demonstrated that monkeys acquire solid homologous protection after infection with live type 3 pneumococci. Efficacy with monovalent type 3 polysaccharide is being studied by the challenge of immunized monkeys.

The immunoelectro osmophoresis procedure has been adapted for serodiagnosing pneumococcal infection. The test appears to be highly sensitive for measuring circulating pneumococcal polysaccharide. The lower limit of detection varies between 0.1 - 10 ug polysaccharide per ml. The utility of this electrophoretic method will be explored further as a procedure for making a type specific diagnosis of pneumococcal antigenemia.

Significance to NIAID Programs and Bio-Medical Research: The experience of a large open population with pneumococcal disease is vital in formulating a polyvalent pneumococcal vaccine for clinical trials to demonstrate efficacy. The development of animal models to demonstrate protection following a defined schedule of immunization will facilitate the generation of data on biological potency of pneumococcal vaccines.

Proposed Course of Project: These studies will be concluded during the present contract year. The effective termination of the contract is July, 1972.

Date Contract Initiated: May 1971

Current Annual Level: Prior year support

WASHINGTON STATE UNIVERSITY - (NIH-70-2130)

Title: The Influence of Interferon and Interferon Inducers on the Pathogenesis of Feline Panleucopenia, Feline Respiratory Disease Complex, and on Canine Distemper in the Ferret

Contractor's Project Director: Jeffrey C. Duenwald, D.V.M.

Project Officer: George J. Galasso, Ph.D.

Objectives: A series of animal models have been established for diseases which closely mimic viral diseases in man which need to be controlled. Having established baseline data on the effects of interferon and interferon inducers in prevention and/or treatment of these viral diseases, these models will serve as a facility for quickly and efficiently examining new antivirals as they are developed.

Major Findings: The contractor has established an assay system for interferon in the cat and in the ferret. Ferrets have proven very unsatisfactory to the current interferon inducers. However, data is available which indicates that poly I·poly C complexed with DEAE-dextran is a better inducer than poly I·poly C alone. It is also of interest that ferrets have a high hydrolytic capacity in their serum for poly I·poly C. The best dose level of poly I·poly C for induction of interferon has been established in the cat. Kinetic studies indicated a maximum titer 12 hours post-inoculation. The effects of two vaccine strains (panleucopenia and infectious bovine rhinotracheitis) as inducers of interferon in the cat have also been studied. Only the latter, given subcutaneously and not intranasally, gave a late and maybe prolonged interferon response. In the cat, COAM gave a ten- to twenty-fold increase in poly I·poly C induced interferon. This contractor has participated in the isoprinosine evaluation study and found isoprinosine to be ineffective in his models.

Significance to NIAID Programs and Bio-Medical Research: Having firmly established baseline data on the effects of interferon and interferon inducers in these animal models, the program will be better able to directly test new antivirals as they are developed in the selected systems. This will afford the program with a direct means of comparison in determining the relative merits of the various substances and their potential for clinical use.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974.

Date Contract Initiated: June 1, 1970

Current Annual Level: $50,600

WEST SIDE VETERAN'S ADMINISTRATION HOSPITAL, CHICAGO (INTERAGENCY AGREEMENT)

Title: Epidemiologic definition of pneumococcal disease patterns in a high-risk population

Contractor's Project Director: Maurice A. Mufson, M.D.

Project Officer: Richard E. Horton, M.D.

Objectives: 1) Determine the incidence of bacteremic and abactermic pneumococcal pneumonia in the Madison Area Neighborhood (MAN) population with identification of pneumococcal serotypes involved 2) determine the overall incidence of pneumonia among adult patients admitted to the Chicago West Side Veteran's Administration Hospital, the University of Illinois Hospital and the Cook County Hospital with parallel delineations of the epidemiologic characteristics of the patients and the identification of the proportion of such infections attributable to the pneumococcus.

Major Findings: From September 1967 to February 1970, bacteremic illnesses were identified in 359 adult in-patients treated at the Cook County Hospital. Eighty-two percent of patients had pneumonia, 11 percent had pneumonia and and extra-pulmonary focus of infection and 7 percent had only an extra-pulmonary focus, chiefly meningitis. Eight capsular types, 8, 4, 5, 12, 3, 1, 7 and 9 in decreasing frequency, accounted for two-thirds of bacteremia cases and two-thirds of deaths. The overall case fatality rate was 28 percent.

The annual estimated incidence rate for pneumococcal disease in the MAN population from February 1971 to June 1971 was 19.8 cases per 1000 individuals.

Significance to NIAID Programs and Bio-Medical Research: Epidemiolgical studies to define characteristics of pneumococcal disease in selected high-risk populations are essential for identifying target groups that would benefit by immunoprophylaxis. In addition, they provide groups that may serve effectively in field testing candidate polyvalent vaccines.

Proposed Course of Project: After review and evaluation during the present contract year, a decision was made to terminate this project on June 30, 1972.

Date Contract Initiated: July 1, 1972

Current Annual Level: $50,000

MEDICAL COLLEGE OF WISCONSIN - (NIH-69-2070)

Title: Preservation of Mammalian Interferons

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officers: Samuel Baron, M.D.
George J. Galasso, Ph.D.

Objectives: To determine the best methods for the preservation of interferons as standard reference reagents. Human and murine interferons will be studied.

Major Findings: High-titered virus-induced mouse interferon, prepared in L cells and partially purified in various ways, has been freeze-dried under different conditions, with and without stabilizing agents. Variables affecting the lability of freeze-dried interferon preparations have been identified. Interferon has been found to behave unlike all other biological materials routinely lyophilized. The mechanism of this difference would be worthy of grant support. It is expected that a supply of both mouse and human interferon will be prepared for lyophilization and submitted to the Research Resources Branch for distribution.

Significance to NIAID Programs and Bio-Medical Research: The requirements for the use of standardized reference reagents is becoming increasingly important in interferon research as well as other areas of medical research. It is important that such reagents maintain a defined reference of potency and stability over a long period of time or decline at a predictable rate. This contract should furnish information not only of importance to interferon reagent stability but also will supply significant data available to all freeze-dried reference reagent standards.

Proposed Course of Project: This contract is in its terminal year. Lyophilized mouse and human interferon will be made available for distribution as a research reagent. The contract was terminated on May 31, 1972.

Date Contract Initiated: May 29, 1969

Current Annual Level: $35,022

MEDICAL COLLEGE OF WISCONSIN - (NIH-70-2125)

Title: Interferon Assay Systems

Contractor's Project Director: Sidney E. Grossberg, M.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: In order to support and expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay. Toward this end this contract is examining a variety of cell lines and strains to determine the most sensitive. They will also examine nutritive and environmental culture conditions, methods of enhancing the interferon effect, optimal dose of viral challenge and enhancement of detection technique.

Major Findings: This productive contract has developed the following information: (a) identification of two human continuous cell lines reasonably sensitive to interferon; (b) development of a hemagglutinin yield-reduction assay using encephalomyocarditis virus; (c) comparative assays of several diploid and continuous human cell cultures for interferon sensitivity; (d) analysis of some of the environmental variables required for the interferon assay; (e) development of a neuraminidase yield-reduction assay which offers high promise for titration of human interferon as well as interferon from other species, and (f) extension of some of these studies to the assay of murine interferon.

Significance to NIAID Programs and Bio-Medical Research: The methods currently in use to assay interferon are tedious, time-consuming, and are not as reliable as would be desired. It is expected that this contract will yield information which would enable an investigator to do more assays in a shorter period of time with great reliability. This would encourage workers such as chemists to enter into this field as well as assisting the current investigators with their work.

Proposed Course of Project: The contract is in its terminal year. It is anticipated that the contractor will complete the assigned work task by June of 1973.

Date Contract Initiated: June 1, 1970

Current Annual Level: $41,400

UNIVERSITY OF WISCONSIN - (NIH-70-2132)

Title: Two Swine Viral Disease Systems for the Study of Interferon and Interferon Inducers

Contractor's Project Director: B. D. Easterday, D.V.M., Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: The establishment of an animal model system for diseases which closely mimic viral diseases in man which need to be controlled. These models are to be readily available for evaluation of new antivirals as they are developed.

Major Findings: This has been a productive contract which has studied (1) the induction of interferon in the pig with viruses and synthetic inducers; (2) pig protection studies against infection with transmissible gastroenteritis and swine influenza viruses; and (3) the effect of interferon and inducers against these viruses in vitro. Of the viruses tested for induction of serum interferon in pigs, only hog cholera virus (virulent strain) induced substantial interferon titers. The interferon levels so induced were substantial, approximating 160 to 320 units per ml. Substantially lower levels of interferon were induced with poly I·poly C, but in preliminary experiments, these levels were enhanced by the use of COAM. Extensive studies with isoprinosine indicated no effect on the course of transmissible gastroenteritis and swine influenza virus infections in the pig.

Significance to NIAID Programs and Bio-Medical Research: Having firmly established these animal model systems, the program will be able to directly test new antivirals as they are developed in the selected systems. This will afford the program a direct means of comparison in determining the relative merits of the various substances.

Proposed Course of Project: It is anticipated that this project will be continued through June 1974.

Date Contract Initiated: June 18, 1970

Current Annual Level: $47,000

WYETH LABORATORIES, INC. - (NIH-69-2109)

Title: Development of Adenovirus Vaccines

Contractor's Project Director: Ben Rubin, Ph.D.

Project Officer: Robert Chanock, M.D.

Objectives: 1) To prepare live adenovirus vaccines types 1, 2 and 5 in dosage forms suitable for oral administration to infants. 2) Prepare one lot of DNA-free adenovirus type 5 subunit vaccine. 3) Complete the preparation of one lot of adenovirus type 7 subunit vaccine. 4) Complete the safety testing of adenovirus type 3 vaccines.

Major Findings: Inactivated and live,oral type 3 vaccines have been safety tested and are ready for clinical evaluation. These vaccines have a higher titer chan those of other adenovirus types prepared and should be very antigenic.

Adenovirus type 5 subunit vaccine was prepared using the lithium iodide method. When this preparation was run through the Amicon Diafiltration system for the removal of DNA fragments, concentration of the Tween 80 occurred to a final level of 9.89%. The excess Tween 80 must be removed before the safety testing of the vaccine can be completed.

Adenovirus type 4 has been packaged in soft gelatin pellets suitable for pediatric use.

Significance to NIAID Program and Bio-Medical Research: Adenovirus types 4 and 7 are the principal causes of acute respiratory disease in military recruits. These vaccines developed under the contract are safe, potent and effective. Adenoviruses type 1, 2 and 5 are significant causes of respiratory disease in young children.

Proposed Course of Project: The contract was terminated on April 30, 1972.

Date Contract Initiated: Contract PH43-63-80 was initiated on January 9, 1963. The contract number was changed to PH43-68-1316 on May 26, 1968. On April 26, 1969 the contract number was changed to NIH-69-2109.

Current Annual Level: Prior year support

YALE UNIVERSITY - (NIH-70-2123)

Title: The Rapid Radiochemical Assay of Interferon: A Conventional and a Matrix Approach

Contractor's Project Director: Richard A. Goldsby, Ph.D.

Project Officer: George J. Galasso, Ph.D.

Objectives: In order to expedite research in the field of interferon, an antiviral agent which may prove useful in man, a study was undertaken to develop a more rapid, quantitative and standardized assay.

Major Findings: The applicability of the radiochemical measurement of the inhibition of viral RNA synthesis by interferon for a rapid assay of interferon has been demonstrated. Sample handling utilizing complementary matrices has been accomplished permitting the handling of large numbers of samples. The contractor has adapted this procedure to the human system. He has perfected the technique so as to use human foreskin tissue. The method has been perfected to the point where large numbers of human interferon assays can now be done with relative ease.

Significance to NIAID Programs and Bio-Medical Research: The methods currently in use to assay interferon are tedious, time-consuming and are not as reliable as would be desired. Investigators whose work demands large numbers of interferon assays can now use this system and in a short period of time obtain titers with high reliability. This should encourage workers such as chemists to enter into this field as well as in assisting current investigators with their work.

Proposed Course of Project: This contract was terminated on April 25, 1972.

Date Contract Initiated: April 26, 1970

Current Annual Level: (prior year support)

Bellanti, J.A.: Biologic significance of the secretory γA immunoglobulins. Pediatrics 48: 715, 1971.

Bellanti, J.A., Catalano, L.W., Chambers, R.W.: Herpes simplex encephalitis: Virologic and serologic study of a patient treated with an interferon inducer. J. Ped. 78: 136-145, 1971.

Bellanti, J.A., Smith, C.B., Kim, H.W., Mills, J., Gerin, J.L., Parrott, R.H. Chanock, R.M.: Serum and local respiratory antibody responses following infection with Mycoplasma pneumoniae or respiratory syncytial virus. U.S. Government Printing Office; 183-194, 1971.

Cavallaro, J.J., Monto, A.S.: HL Cells: A sensitive line for the isolation and propagation of respiratory syncytial virus. Proc. Soc. Exp. Biol. Med. "In Press".

Charnock, E.L., Cramblett, H.G.: 5-Iodo-2'deoxyuridine in neonatal herpesvirus hominis encephalitis. J. Ped. 76: 459-463, 1970.

Chow, A.W., Foerster, J., Hryniuk, W.: Cytosine arabinoside therapy for herpesvirus infections. Antimicrob. Agents and Chem. 214-217, 1970.

Chowchuvech, E., Weissenbacher, M., Schmunis, G., Sawicki, L., Galin, M.A., Baron, S. The influence of polyinosinic-polycytidylic acid complex on experimental acute toxoplasmic retino-choroiditis in rabbits. Inv. Ophth. 2: No. 3, 182-188, March, 1972.

Cooper, L.Z.: Rubella: The problem. PAHO, Washington, D.C. Scientific Publication No. 226; 229-239, 1971.

Cooper, L.Z.: Rubella vaccination with special emphasis on immunization of women. Canad. J. of Pub. Health 62: 48-55, Sept. 1971.

Cooper, L.Z., Florman, A.L., Ziring, P.R., Krugman, S.: Loss of rubella HI antibody in children with congenital rubella. Amer. J. of Dis. of Child. 122: 397-403, Nov. 1971.

Couch, R.B., Douglas, R.G., Jr., Fedson, D., Kasel, J.: Protection against experimental influenza in man. III. J. Infec. Dis. 124: 473-480, Nov. 1971.

Dean, A.G., Couch, R.B., Douglas, R.G., Jr., Jones, T.C., Knight, V.: Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. III. Microbiologic investigations and human volunteer experiments. Amer. J. Epid. "In Press".

Detels, R., Kim, K., Gale, J.L., Grayston, J.T.: Viral shedding in Chinese children following vaccination with HPV-77 and Cendehill-51 live attenuated rubella vaccines. Amer. J. of Epid. 94: (5) 473-478, 1971.

Douglas, R.G., Jr., Couch, R.B.: Parenteral inactivated rhinovirus vaccine: Minimal protective effect. Proc. Soc. Exp. Biol. Med. March, 1972 139: No. 3, 899-902.

Edmondson, W.P., Jr., Rothenberg, R., White, P.W., Gwaltney, J.M., Jr.: A comparison of subcutaneous, nasal and combined influenza vaccination. II. Protection against natural challenge. Amer. J. of Epid. 93: No. 6 480-486; June, 1971.

Foy, J.M., Cooney, M.K., McMahan, R., Bor, E., Grayston, T.J.: Single dose monovalent A_2/Hong Kong influenza vaccine. Efficacy 14 months after immunization. JAMA 217: No. 8, 1067-1071, August 23, 1971.

Foy, H.M., Kenny, G.E., McMahan, R., Kaiser, G., Grayston, J.T.: Mycoplasma pneumoniae in the community. Amer. J. of Epid. 93: 55-67, 1971.

Foy, H.M., Nugent, C.G., Kenny, G.E., McMahan, R., Grayston, J.T.: Repeated Mycoplasma pneumoniae after 4 1/2 years. JAMA 216: No. 4, 671-672; April 26, 1971.

Green, Maurice: Transcription of viral genes in cells transformed by DNA and RNA tumor viruses. Papanicolaou Cancer Res. Inst. Sym. Nucleic Acid Syn. in Viral Infection D.W. Ribbons, J.F. Woessner, J. Schultz, Editors.

Green, Maurice, Hodap, Magdalena: Asymmetric transcription of early adenovirus genes by the Escherichia coli RNA polymerase in vitro. J. Molec. Biol. 64: 305-309, 1972.

Green, M., Parsons, J.T., Caffier, H., Landgraf-Leurs, M., Tsuei, D.: Transcription of adenovirus genes in productively infected and in transformed cells The Biology of Oncogenic Viruses Amsterdam, The Netherlands, North-Holland Publishing Co. 88-97, 1971.

Green, M., Rokutanda, M., Fujinaga, K., Rokutanda, H., Gurgo, C., Ray, R.K., Parsons, J.T.: Molecular and submolecular programming of viral oncogenesis. Human Tumor Virology and Immunology (U. of Tokyo Press) 1970.

Green, M., Rokutanda, M., Fujinaga, K., Rokutanda, H., Gurgo, C., Ray, R.K., Parsons, J.T.: Synthesis of DNA by RNA tumor viruses and viral RNA by virus transformed cells. The Biology of Oncogenic Viruses L. Silvestri, Editor North-Holland Publishing Co.

Gwaltney, J.M., Jr.: Antiviral chemotherapy. Va. Med. Monthly July, 1971, 368-370.

Gwaltney, J.M., Jr.: Influenza vaccine. JAMA 217: 333-334, July, 1971.

Gwaltney, J.M., Jr.: Virology of middle ear. Ann.of Otol., Rhin., & Laryn. 80: No. 3, 365-570; June, 1971.

Gwaltney, J.M., Jr., Edmondson, W.P., Jr., Rothenberg, R., White, P.W.: A comparison of subcutaneous, nasal and combined influenza vaccination. I. Antigenicity. Amer. J. Epid. 93: No. 6, 472-479, June, 1971.

Halstead, S.B., Diwan, A.R.: Failure to transmit rubella virus vaccine. A close-contact study in adults. JAMA 215: No. 4, 634-636, Jan. 25, 1971.

Heggie, Alfred D.: Pathogenesis of the rubella exanthem: Detection of rubella virus in the skin. New Engl. J. Med. 285: 664-666, Sept. 16, 1971.

Hendley, J.O., Edmondson, W.P., Gwaltney, J.M., Jr.: Relation between naturally acquired immunity and infectivity of two rhinoviruses in volunteers. J. Inf. Dis. 125: No. 3, 243-247, 1972.

Horstmann, D.M., Liebhaber, H., Kohorn, E.I.: Post-partum vaccination of rubella-susceptible women. Lancet 1003-1006, Nov. 14, 1970.

Horstmann, D.M., Liebhaber, H., Le Bouvier, G.L., Rosenberg, D.A., Halstead, S.B.: Rubella: Reinfection of vaccinated and naturally immune persons exposed in an epidemic. New Engl. J. Med. 283: 771-778, October 8, 1970.

Hsiung, G.D., Swack, N.S., Gharpure, N., Tscholl, K.: A comparative study of cytomegaloviruses of primates and nonprimates. Med. Primatology 709-720 1970.

Isacson, P., Kehrer, A.F., Wilson, H., Williams, S.: Comparative study of live, attenuated rubella virus vaccines during the immediate puerperim. Obstet. and Gyn. 37: No. 3, 332-337, March, 1971.

Kasel, J.A., Couch, R.B., Douglas, R.G., Jr.: Antigenicity of alum and aqueous adenovirus hexon antigen vaccines in man. J. Immun. 107: No. 3, 916-919, Sept., 1971.

Kenny, G.E.: Immunogenicity of Mycoplasma pneumoniae. Infec. and Immun. 3: No. 4, 510-515, April, 1971.

Kim, H.W., Arrobio, J.O., Pyles, G., Brandt, C.D., Camargo, E., Chanock, R.M., Parrott, R.H.: Clinical and immunological response of infants and children to administration of low-temperature adapted respiratory syncytial virus. Pediatrics 48: 745-755, 1971.

Knight, J., Couch, R.B., Douglas, R.G., Jr., Tauraso, N.M.: Serologic response and natural infectious challenge of recipients of zonal ultracentrifuged influenza A/2 Aichi/2/68 vaccine. Bull. WHO "In Press".

Leedon, J.M., Wilkins, J., Portnoy, B., Salvatore, M.A.: Commentary: Important assumptions, extrapolations, and established facts which underlie the use of live rubella virus vaccines. Amer. J. Epid. 92: No. 3, 151-161, 1970.

Le Bouvier, G.L., Plotkin, S.A.: Precipitin responses to rubella vaccine RA 27/3. J. Inf. Dis. 123: No. 2, 220-223, Feb. 1971.

Leibhaber, H., Ingalls, T.H., Le Bouvier, G.L., Horstmann, D.M.: Persistence of immunity and resistance to challenge two years after vaccination with RA 27/3 rubella vaccine. Amer. J. Dis. of Child. 123: 133-136, Feb. 1972.

Leibovitz, A., Coultrip, R.L., Kilbourne, E.D., Letgers, L.J., Smith, C.D., Chin, J., Schulman, J.L.: Correlated studies of a recombinant influenza-like vaccine. IV. Protection against naturally occurring influenza in military trainees. J. Inf. Dis. 124: No. 5, 481-487, Nov. 1971.

Lerman, S.J., Nankervis, G.A., Heggie, A.D., Gold, E.: Immunologic response, virus excretion, and joint reactions with rubella vaccine. A study of adolescent girls and young women given live attenuated virus vaccine (HPV-77:DE-5). Ann. Int. Med. 74: No. 1, 67-73, Jan. 1971.

Maletzky, A.J., Cooney, M.K., Luce, R., Kenny, G.E., Grayston, J.T.: Epidemiology of viral and mycoplasma agents associated with childhood lower respiratory illness in a civilian population. J. Ped. 78: No. 3, 407-414, 1971.

Moehring, J. M., Forsyth, B.R.: The role of the interferon system in respiratory syncytial virus infections. Proc. Soc. Exp. Biol. Med. 138: No. 3, 1009-1014, December, 1971.

Mogabgab, W.J., Stowe, F.R. Jr.: Evaluation of attenuated rubella virus vaccine in families. Amer. J. Dis. of Child. 122: 122-128, August, 1971.

Monto, A.S., Cavallaro, J.H.: The Tecumseh study of respiratory illness. II. Patterns of occurrence of infection with respiratory pathogens, 1965-1969. Amer. J. Epid. 94: 280, 1971.

Monto, A.S., Lim, S.K.: The Tecumseh study of respiratory illness. III. Incidence and periodicity of respiratory syncytial virus and Mycoplasma pneumoniae infections. Amer. J. Epid. 94: 290, 1971.

Monto, A.S., Napier, J.A., Metzner, H.L.: The Tecumseh study of respiratory illness. I. Plan of study and observations on syndromes of acute respiratory disease. Amer. J. Epid. 94: 269, 1971.

Nahmias, A.J., Alford, C.A., Korones, J.B.: Infection of the newborn with herpesvirus hominis. Adv. in Ped. 17: 185-226, 1970.

Oroszlan, S., Stanley, T.B., Jr., Gilden, R.V.: Communications: Purification and serologic tests with adenovirus 2 virion and T antigens. J. Immun. 104: No. 3, 759-761, March, 1970.

Parsons, J.T., Green, M.: Biochemical studies on adenovirus multiplication. XVIII. Resolution of early virus-specific RNA species in Ad 2 infected and transformed cells. Virology 45: 154-164, 1971.

Raskas, H.J., Green, M.: DNA-RNA and DNA-DNA hybridization in virus research. Meth. in Virol. N.Y. Academic Press, 5: 247-269, 1971.

Rossen, R.D., Kasel, J.A., Couch, R.B.: The secretory immune system: Its relation to respiratory viral infection. Melnick, J., Melnick, M.B., Editors. Prog. in Med. Virol. 13: 194-238, 1971.

Schachter, N., Galin, M.A., Baron, S., Billiau, A., Weissenbacher, M.: Comparison of antiviral action of interferon, interferon inducers and IDU against herpes simplex and other viruses. Ann. Ophth. 2: 795, 1970.

Somerson, N.L., Senterfit, L.B., Couch, R.B., Robillard, N., Pollack, J.D.: An improved M. pneumoniae vaccine. Bull. WHO "In Press".

Tint, H., Bierly, M.Z.: The clinical evaluation of the CV1 vaccinia strain: Background and status. Proc. Symp. on Smallpox, Yugoslav Acad. of Sciences and Arts, Zagreb, 1969 167-179.

Tsuei, D., Fujinaga, K., Green, M.: Mechanism of viral carcinogenesis by DNA mammalian viruses: RNA transcripts containing viral and highly reiterated cellular base sequences in adenovirus-transformed cells. Proc. Nat. Acad. Sci. USA, 69: No. 2, 427-430, February, 1972.

Vernon, S.K., Stasny, J.R., Neurath, A.R., Rubin, B.A.: Electron microscopy of DNA from adeno-associated virus type 1. J. Gen. Virol. 10: 267-272, 1971.

Wittner, M.I., Fox, E.N.: Micro complement fixation assay for type-specific group A streptococcal antibody. Inf. and Immun. 441-445, October, 1971.

Ziring, P.R.: Current status of the rubella problem. Cardiovascular Nursing 6: 47-50, July-August, 1970.

Ziring, P.R., Florman, A.L., Cooper, L.Z.: The diagnosis of rubella. Ped. Clinics of N. America, 18: 87-97, 1971.

Publications - B

Galass, G.J., Youngner, J.S., Glasgow, L.A.: Antiviral research in the Soviet Union. J. Infec. Dis. 125: 455-456, 1972.

Glasgow, L.A., Galasso, G.J. (Editors): Lack of antiviral activity of isoprinosine in experimental model infections. "In Press".

Madden, D.L., Horton, R.E., McCullough, N.B.: Exposure of germfree and conventional chickens to the SA serotype of avian Mycoplasma. Avian Dis. 16: No. 2, January-March, 1972.

Mullally, D.I.: Future vaccines - prospects and problems. Devel. in Indus. Microb. 11: Chap. 2, 12-15, 1970.

TRANSPLANTATION AND IMMUNOLOGY PROGRAM

1. Summary

Histocompatibility typing is a complex procedure requiring multiple serological tests to identify the antigens involved in the rejection of transplanted organs. Program contractors have demonstrated the importance of platelet complement fixation and mixed lymphocyte culture techniques. The validity of typing is improved by the utilization of reliable antisera and multiple procedures by well-qualified laboratories. Validity of reagents being distributed by the program is insured by testing in quality control laboratories and a continuing review of the results of typing in over 80 U.S. laboratories using a standardized technique with identical reagents. Considerable program effort has been devoted to improve techniques for freeze drying of reagents. The present systematic program for processing of frozen serum will result in a majority of the serum becoming available in a stable form which can be shipped at ambient temperatures.

The Transplantation and Immunology Branch sponsors contracts to evaluate drugs and antilymphocyte serum used as immunosuppressants. Sub-human primate skin graft studies indicate that rabbit antihuman thymocyte serum is very effective. Large quantities of this material are being produced for use in a clinical program during the next fiscal year.

Another essential part of the Transplantation Program is the preservation of cells and organs. The program has sponsored studies of kidneys. Quantities of purified HL-A and H-2 soluble antigens have been produced; HL-A antigens are used by a number of investigators in studies to induce tolerance in potential recipients of organ transplants. HL-A antigens are also being used to immunize laboratory animals to produce high-titer antisera needed for histocompatibility typing.

One contract studies the immunologic phenomena associated with organ rejection in the patient. This indicates that there is an increase in rheumatoid-like factor in transplant recipients and that antigen-antibody complexes may be deposited in the kidney and other organs in these patients.

One of the most important functions of the Transplantation and Immunology Program is to collect and disseminate information on reagents, typing techniques, organ transplants and the efficacy of tissue matching. The program supports registries, sponsors workshops and scientific meetings and, through the publication of contractor's reports and the issuance of catalogs, typing manuals and periodic statistical reports, provides a valuable resource of scientific and technical information.

2. Program Status Report

During the period July 1, 1971 to June 30, 1972, the Branch provided approximately $1.8 million in contract funds for the support of transplantation and immunology programs. The research and development efforts of the Branch can be considered in six general categories.

1. Histocompatibility Typing Facilities and Immunologic Reagents

Since the NIAID Collaborative Transplantation Immunology Program was established in 1965, much progress has been made in developing the methodology of histocompatibility typing and the reagents necessary for this work. The current emphases of the ten contracts in this field are to evaluate the efficacy of typing in selected donors and recipients for organ transplantation; to provide a continuing supply of high-quality typing sera both in bulk and in micro-quantities on a standardized tray; to develop new typing techniques that are potentially simpler or more accurate than those currently employed; to conduct research directed at discovering new specificities; and to provide other reagents used in histocompatibility research. These ten contracts have been supported to work in one or more of the above areas, with a total FY'72 funding of $467,388.

Although the utility of tissue typing has been demonstrated in matching related donors and recipients there still remains the problem of evaluating the efficacy of these procedures in the selection of appropriate cadaver kidney donors. Well-matched donor-recipient pairs seem to have fewer episodes of threatened graft rejection than recipients receiving kidneys from completely mismatched donors. The analyses of the data based on a simple count of mismatched antigens have yielded confusing results due in part to the difficulties in collecting accurate clinical and typing data. The consensus to date is that there exists a correlation between closeness of matching and renal graft function. Earlier results from more sophisticated analyses based on the numbers of shared haplotypes between donors and recipients have been encouraging. One contract is analyzing the usefulness of typing in bone marrow transplantation. Through the use of a standardized tray and uniform cytotoxicity typing techniques 90 U.S. laboratories are participating in a program to collect uniform typing data. A continuing program of quality control, both of reagents and the reproducibility of laboratory results is underway. Over 1,500 typings are being performed monthly utilizing the standardized trays and techniques.

The NIAID typing serum bank currently contains over 1,200 sera with a total volume of 227 liters. Additions to the bank in FY'72 included 75 (23,716 ml.) sera obtained by contracts from five laboratories, 99 (25,653 ml.) donated by users of the bank and 44 (88,919 ml.) purchased outright by the Branch. Quality control analyses of the

sera prior to their procurement were performed by six typing laboratories; this system has increased the overall quality of the additions to the bank. Usage of the bank remained heavy with 940 shipments (24,514 ml.) dispensed to 302 investigators. In addition, significant improvements in the clinical typing tray program were made. Fifty-five sera were carefully selected for use on a tray to be kept constant over a two-year period; typing results are collected from each tray and will be used in an expansion of the Branch's effort to evaluate the efficacy of typing. Approximately 1,500 trays per month are made on one contract and are being used by U.S. clinical typing programs. New techniques of typing were studied on four contracts. The use of kidney cells has been found to provide a valuable additional histocompatibility cross-match test and they may help detect additional antigens in clinical typing. The platelet complement fixation test has been shown to be a simple, easily readable typing technique which is being automated. Neutrophil-specific antigens have been discovered and are being studied in bone-marrow transplantation donor-recipient pairs. Better methodology and reagents have resulted in the discovery of new antigens; several of the typing contracts have made contributions in this area.

Two contracts have produced reagents other than typing sera used in histocompatibility research. Human complement components and inhibitors are distributed to qualified investigators, and mouse spleens have been provided in large quantities as starting material for the production of soluble mouse histocompatibility antigen (described in Section IV).

II. Studies of Immunosuppression

Four contracts totaling $676,274 provides FY'72 funds to evaluate potentially active immunosuppressive drugs and sera in animals and patients. These help to develop techniques for the prevention or the abrogation of graft rejection. Cyclophosphamide and a variety of new antineoplastic drugs are currently being investigated. Studies to date in animals and patients indicate that cyclophosphamide is an effective agent for the prevention of graft rejection in bone marrow transplantion. Other antitumor agents (methotrexate, cytosine arabinoside and L-asparaginase) were used individually and with antilymphocyte serum and have been shown to be effective in preventing first and second set graft rejection. Important methodology has been developed to evaluate immunosuppressives.

Antilymphocyte serum (ALS) and purified antilymphocyte globulin (ALG) are of the current interest as means of preventing and reversing rejection. Over 35 percent of the U.S. transplantation programs utilize these preparations. Unfortunately, few controlled studies have been conducted to evaluate the efficacy of these products. The Transplantation and Immunology Branch has established the only Western Hemisphere facility for the mass *in vivo* testing of ALS

and ALG. This contract has screened more than 130 preparations, many of which are currently being used in the clinic. The two most effective antigens for the production of ALS are thoracic duct and thymus cells; ALS raised in rabbits is superior to that produced in horses in the prolongation of Rhesus and chimpanzee skin grafts. Preclinical pharmacology and toxicity studies are underway on a product which will be used in a 50-patient clinical trial. The high cost of subhuman primate testing has prompted the search for effective in vitro techniques of assaying ALS and ALG. Studies indicate that rabbit antihuman thymocyte serum is effective only if it exhibits a high in-vitro lymphocytotoxic titer. This technique is being utilized to exclude non-effective rabbit antisera from the pool of material being readied for clinical trial.

III. Organ Preservation Studies

A transplantation program is heavily dependent upon the availability of viable organs. Because of the many difficulties involved in the long-term preservation of organs, current kidney-sharing programs maintain a pool of potential recipients from which one is selected (via histocompatibility matching) to receive a specific kidney immediately after the organ becomes available.

If long-term kidney preservation were possible, a pool of organs could be maintained from which one could be selected for a specific patient as soon as that patient needed such a transplant. An NIAID contract has been responsible for the development of a multifaceted attack on the problems of long-term organ preservation. An accomplishment includes the development of cryoprotective media more effective than those currently used; the application of methods of treatment used against frostbite to the resuscitation of cold organs; the development of an enzyme assay for organ damage incurred during freezing and thawing, a microwave device which rapidly and evenly thaws a kidney; and the use of hyperbaria to decrease the formation of extra-cellular ice during the freezing process.

IV. Antigen Studies

The Institute provides $140,000 in FY'72 for the support of two contracts for the study of soluble murine histocompatibility antigens. Several methods of antigen solubilization and fractionation are being employed to produce active stable fractions which are then assayed by a variety of in vitro and in vivo techniques. These antigen fractions are also provided to NIH intramural, contract and grantee investigators who aid in the investigation of these products for their immunogenic and tolerogenic properties. These contracts have established the methodology for the production of quantities of soluble antigens for laboratory investigation.

V. Immunological Phenomena

Two contracts receiving no FY'72 funds (annual level of approximately $160,000) evaluate the immunological changes in the tissues and serum of patients and animals which have received organ transplants and/or are immunosuppressed. There is an increase in rheumatoid-like factor in the serum of transplant recipients and titers usually are higher in patients who have incompatible grafts. The mechanism of hyperacute graft rejection and its immunological process is under investigation. Immunohistological changes in transplanted and rejected renal grafts are being investigated to assess whether graft rejection is due to recurrent nephritis, to the deposition of circulating immune complexes in the renal tubules or glomeruli, or to an immunologic histoincompatibility reaction. Studies involving antilymphocyte globulin (ALG) preparations used in immunosuppressive therapy have revealed that many of those preparations derived from solid lymphoid organ lymphocytes contained anti-glomerular basement membrane (GBM) antibodies.

VI. Statistical Studies and the Dissemination of Scientific Information

The Branch supports part of the American College of Surgeons/National Institutes of Health contract for the collection of transplantation information. The Branch Chief is the Project Officer for this contract.

The Transplantation and Immunology Branch conducts its own program for the collection of fundamental information on transplants. On an annual basis, serum users are asked to provide data indicating the numbers of transplants of different organs conducted by the various hospitals for whom they provide typing services. The data are collated and used by the Branch in assessing priorities for the distribution of serum, and distributed to other serum users and sent to the ACS/NIH Registry. Experience in the past has shown that this mechanism has been far more successful in obtaining transplantation information than other registries. During 1970, serum users indicated that 3,900 kidney transplants had been performed in 300 hospitals around the world. As part of its information program, the Branch prepares an annual updating of the Tissue Typing Catalog. The last issue of this catalog was distributed in November 1971 to approximately 700 users. This catalog, which is over 800 pages in length, contains information on 1,200 sera which are available from the Institute's bank. It lists the availability of serum and a great deal of information concerning the specificity, utility, titers and the frequency of reaction with the various racial groups. It is the only such document available for use by transplantation immunologists. The Manual of Tissue Typing Techniques was revised during the last fiscal year. This contains up to the minute information on the various tests which may be used for histocompatibility matching, and is distributed to investigators and used in the tissue typing courses. The Branch also provides catalogs of other reagents, including H-2 (mouse) antisera, complement components, and soluble antigen fractions.

Contract funds will provide partial support for the 4th International Congress of the Transplantation Society held at San Francisco in September 1972. Partial support was also provided for the 1st International Immunology Congress held in Washington in August 1971. Funds were provided for a three-day meeting in Dallas, Texas of the principal typers and transplant teams to discuss the organization of a nationwide program to collect and evaluate typing and clinical data on renal transplants. Under program auspices, a three-day meeting of immunologists was held at Brook Lodge, Michigan to discuss altered immune responsiveness. These conferences have been instrumental in helping the Institute in planning future programs.

With contract support from the program, the WHO Expert Committee on Histocompatibility conducted studies of antigens in racial isolates to search for and determine the frequency of exotic lymphocyte antigens. As the result of these studies, discussed at a workshop in Evian, France, valuable new information was developed pertaining to new antigens and "splits" of currently recognized antigens. This information will lead to a better understanding of tissue typing.

The Branch also distributes information to scientists by means of periodic bulletins, and aids in the organization and operation of histocompatibility workshops.

3. CONTRACT NARRATIVES

ALBERT EINSTEIN COLLEGE OF MEDICINE, YESHIVA UNIVERSITY (PH43-68-1295)

Title: Preparation of soluble purified H-2 isoantigens

Contractor's Project Director: Dr. Stanley G. Nathenson

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contract provides soluble mouse H-2 antigens to selected qualified investigators who are interested in applying these antigens in studies of immunogenicity and immunological tolerance. This contract provides the contractor with funds for storage and distribution of these antigens and to improve the efficiency of the solubilization and purification processes.

Major Findings: This investigator has processed approximately 200,000 mouse spleens from seven different strains of mice to acquire soluble H-2 histocompatibility antigens in various stages of purity. Thus far, forty investigators have utilized approximately 1,900 milligrams of these soluble antigenic fractions in support of their research activities. These soluble H-2 alloantigens have been shown to be immunogenic as well as modify graft-versus-host reactions.

Significance to NIAID Program and Bio-Medical Research: Basic immunogenetic knowledge concerning the immunogenicity and tolerogenicity of mouse H-2 soluble histocompatibility antigens and their relationship to accelerating and inhibiting graft rejection will be enhanced by this contract. These studies are in concert with similar studies of human HL-A soluble antigen production and eventual immunogenicity and tolerogenicity studies in experimental animals and humans. The ultimate goal is to develop methods to abrogate the graft rejection process and permit permanent graft survival.

Proposed Course of Project: Termination December 23, 1972. This contract is being terminated because the current inventory of soluble H-2 antigen is sufficient to allow new investigators to acquire these as standard preparations. The methodology has been published and numerous investigators are now preparing their own soluble H-2 antigen.

Date Contract Initiated: June 24, 1968

Current Annual Level: $45,000

ALBERT EINSTEIN COLLEGE OF MEDICINE (NIH69-2071)

Title: Human neutrophil-specific antibodies

Contractor's Project Director: Dr. Parviz Lalezari

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor is providing the Institute Serum Bank with human neutrophil-specific antisera and is searching for new such antisera through a program of screening potential donors. Once obtained, he is measuring the activity of these antisera against neutrophils and lymphocytes. These antisera are used clinically by the contractor and by a growing number of other investigators in conjunction with bone marrow transplantation.

Major Findings: Neutrophil-specific antigens have been shown to be rare but are of clinical significance in neonatal neutropenia and have been implicated as a factor in the rejection of bone marrow grafts. Women with infants having neonatal neutropenia have been shown to be the only practical source of these antisera in high titer, and among this population the contractor has uncovered the four distinct neutrophil specificities which are known at present, and has contributed each in quantity to the NIAID Serum Bank. Two of the antigens are apparently alleles. Solubilization of one of the antigens has been accomplished and the chemical identification of each antigen is under investigation.

Significance to NIAID Program and Bio-Medical Research: The investigation of neutrophil-specific antigens is a field of research in which this contractor is the sole participant. Evidence is accumulating from many sources that these antigens may well be of great importance in determining the success or failure of bone marrow transplants, which are being performed in increasing number for severe leukopenia of various etiologies. The activity of these antigens against renal or other solid organ transplants is also under study. Definition of the genetics of neutrophil antigens and the further determination of their pathological significance is of pressing clinical importance to transplantation and undoubtedly to medical immunology in general.

Proposed Course of Project: Continuation for a final year through December 26, 1972

Date Contract Initiated: June 27, 1969

Current Annual Level: $44,000

UNIVERSITY OF CALIFORNIA, LOS ANGELES (PH43-65-994)

Title: Evaluation of the role of leukocyte typing in kidney transplantation

Contractor's Project Director: Dr. Paul I. Terasaki

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: A major aim of this contract has been to evaluate the importance of histocompatibility tissue typing in clinical transplantation within the Los Angeles area through the use of lymphocytotoxicity, platelet complement fixation, and mixed lymphoycte culture techniques. The contractor has also evaluated antisera which has been submitted by the NIAID as candidates for the Serum Bank, and he himself conducts a screening program for valuable antisera. He has prepared NIH Typing Trays, which contain sera covering a full range of specificities, for distribution by the Branch to clinical tissue typing laboratories.

Major Findings: The NIH Typing Trays, which were devised and developed through this contract, have become the major source of quality typing sera for clinical typing laboratories in the United States and, as currently standardized through another contractor, they will continue to serve as a source of highly comparable typing data over the next several years. The reagent stock of the Serum Bank has been improved and supplemented through the quality control of this contractor. Some valuable serum has been obtained and new specificities defined through his screening. The contractor's comparisons of typing techniques, specifically lymphocytotoxicity, platelet complement fixation, and mixed lymphocyte culture, have helped to determine the advantages and limitations of each technique. Correlations of typing results with the clinical outcome in transplant patients are still embroiled in controversy. This contractor has raised some question as to the relevance to renal graft survival of matched vs. unmatched organs; his doing so has underscored the need for a definitive study of this issue and this is now being pursued by the Branch.

Significance to NIAID Program and Bio-Medical Research: This contract has aided the Branch in establishing a coordinated system of tissue typing through the NIH Tray, making possible the correlation of typing data between separate transplant centers. His elucidation of typing techniques has contributed to the effort to make tissue typing more accurate and rapid; these are particularly important goals in clinical typing. Maintaining the high standards of the NIAID Serum Bank reagents is also due in part to the quality control efforts of this contract.

Proposed Course of Project: Terminated September 1, 1971

Date Contract Initiated: June 22, 1965

Current Annual Level: Prior year support

CLEVELAND CLINIC FOUNDATION (PH43-68-1298)

Title: Human tissue typing and transplantation laboratory

Contractor's Project Director: Dr. William E. Braun

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor maintains a histocompatibility typing facility for the Cleveland area which provides the NIAID Serum Bank with high quality HL-A antisera through a screening program. In addition, he serves as a quality control laboratory for typing reagents. He is also involved in the comparison of two variations of lymphocytotoxicity typing: one utilizing peripheral blood lymphocytes, the other using lymph node as the source of cells to be typed.

Major Findings: This contractor has developed an efficient typing program and has collected a panel of high quality antisera to be used in conjunction with the NIH tray in clinical typing. The quality of his work is high and for this reason he has become a major quality control laboratory for the identification of new sera for the NIAID Serum Bank. He also evaluates random samples of freshly produced NIH tray in clinical typing. The quality of his work is high and for this reason he has become a major quality control laboratory for the identification of new sera for the NIAID Serum Bank. He also evaluates random samples of freshly produced NIH trays to insure their quality. The contractor's own antiserum screening program, involving primarily multiparous women, has itself provided the Serum Bank with several valuable antisera. His clinical experience accumulated thus far largely from related donors and recipients supports data from other laboratories showing better graft survival with matched than with mismatched renal transplants. In addition, he has found it practical to uncover apparently hidden HL-A antigens by trypsinzation of the cells and is continuing his investigations into this potentially valuable technique.

Significance to NIAID Program and Bio-Medical Research: This contract has contributed greatly to the efforts of the Branch to assess the efficacy of tissue typing in selecting organ donors and recipients. It has been especially productive in the procurement of new sera, essential for the operation of the Serum Bank. The Contractor's highly reliable performance in typing is basic to his quality control function, an essential part of the Branch's effort to constantly improve the serum reagent stock of the Bank.

Proposed Course of Project: Continuation through December 27, 1972

Date Contract Initiated: June 28, 1968

Current Annual Level: $69,861

COMMUNITY BLOOD COUNCIL OF GREATER NEW YORK (PH43-67-1334)

Title: Histocompatibility testing of donors and recipients of organ grafts.

Contractor's Project Director: Dr. Fred H. Allen, Jr.

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor is engaged in the evaluation of tissue typing as a means of selecting the donors and recipients of organ transplants within the New York City area.

Major Findings: This contractor has utilized a two stage lymphocytotoxicity test and a panel of 56 sera to conduct tissue typing on a 24 hour basis in the New York City region. Using this method they have accomplished as many as 900 typings annually, representing approximately 50 renal transplants performed. Data on a limited number of transplants, some dating back as far as three years, suggest a longer survival of well-matched grafts if the donors and recipients. Their data has helped to rule out any possibility of close genetic linkage between most of the common RBC antigens and the HL-A antigen system.

Significance to NIAID Program and to Biomedical Research: This contract affords an opportunity not only to determine the importance of histocompatibility "leukocyte" typing in the selection of donors and recipients of organ grafts, but also to correlate RBC and serum protein groups with the results of typing and the course. The study of the efficacy of typing in selecting unrelated graft donors to recipients is encouraging when viewed by the haplotype analysis, and may be even more so as larger numbers of patients can be studied.

Proposed Course of Project: Terminates October 31, 1972

Date Contract Initiated: June 29, 1967

Current Annual Level: Prior year support ($53,400)

HARVARD UNIVERSITY (NIH-69-90)

Title: H-2 histocompatibility antigen extraction, purification and characterization

Contractor's Project Director: Dr. Paul S. Russell

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor is to produce water soluble H-2 antigens from mouse tissue and characterize the reactions of these antigens by determining their in vitro and in vivo biological activity. Sufficient quantities of solubilized antigen are to be produced so that they can be distributed to qualified investigators as directed by the project officer. Additionally, this contractor is to conduct comparative in vivo biological tests with other contract produced soluble H-2 antigens.

Major Findings: This contractor has produced sufficient quantities of soluble H-2 antigens and thus far seven qualified investigators have requested and received these antigens. These antigens have been shown to be immunogenic and they have enhanced skin graft survival in congenic strains of mice. This contractor has been successful in separating the d and k region antigens from solubilized mouse tissue and in vivo biological tests have shown that the k region antigens possess greater immunogenic properties than the d region antigens.

Significance to NIAID Program and Bio-Medical Research: The mouse tissue histocompatibility antigens (H-2) are needed in the Transplantation and Immunology Branch program to learn more about their relationship to the human (HL-A) histocompatibility antigens and the method of inducing tolerance in mice and men thereby avoiding the graft rejection process. The methods of extraction and purification of soluble HL-A and H-2 antigens are similar but the in vivo testing for graft prolongation can better be accomplished first in the well-defined immunogenic mouse model.

Proposed Course of Project: Termination on May 8, 1973. The question of acquiring tolerance or immune enhancement with these soluble H-2 antigenic preparations should be answered by the end of this contract.

Date Contract Initiated: April 9, 1969

Current Annual Level: $112,669

IRWIN MEMORIAL BLOOD BANK OF THE SAN FRANCISCO MEDICAL SOCIETY (PH43-68-945)

Title: Operation of a Regional Leukocyte Phenotyping Facility in a Community Blood Bank

Contractor's Project Director: Dr. Herbert A. Perkins

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor has conducted histocompatibility typing of leukocytes and kidney cells for renal transplantation in the San Francisco area. He conducts a screening program to provide histocompatibility typing antisera for the NIAID Serum Bank, and serves as a quality control testing laboratory to evaluate candidate sera for the Bank. In addition, this contractor is comparing the techniques of lymphocytotoxicity and fluorochromasia in the matching of prospective donors and recipients of human organ transplants.

Major Findings: Using the fluorochromasia technique, and more recently also the lymphocytotoxicity technique, this contractor has typed for over 100 renal transplants during FY '72. The studies of kidney cell typing by the former technique have occasionally uncovered HL-A factors and incompatible cross matches which were missed with the use of lymphocytes. Graft survival in siblings has correlated very well with haplotype matching, and even in the case of cadaver-donor transplants it appears that survival does improve when HL-A incompatibilities are minimized, especially when the analysis is limited to "full-house" donor grafts. Long-term storage of typed cells in liquid nitrogen to permit retrospective typing has become routine at this laboratory; this practice often allows more complete typings, and permits a more thorough retrospective analysis of the clinical results.

Significance to NIAID Program and Bio-Medical Research: The very active nature of the renal transplant program served by this contractor facilitates his efforts to compare the clinical result with that predicted by the histocompatibility data. This is a question of great importance, and is as yet unanswered His comparison of the fluorochromasia and lymphocytotoxicity techniques contribute to the efforts of the Branch to standardize tissue typing procedures so that results will correlate from region to region. The contractor's service to the NIAID Serum Bank through the provision of antiserum and quality control work is essential to the function of the Bank.

Proposed Course of Project: Continuation through December 27, 1972

Date Contract Initiated: March 27, 1968

Current Annual Level: $112,461

THE JACKSON LABORATORY (PH43-66-483)

Title: The production of monospecific H-2 antisera and specific mouse spleens.

Contractor's Project Director: Dr. George Snell and Dr. Mariana Cherry

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor maintains a mouse H-2 alloantiserum bank which stores and distributes these antisera to NIAID-authorized requesters. He is to periodically check these sera for potency of reactivity and specificity recharacterization. In addition, this contractor prepares, stores and distributes approximately 60,000 mouse spleens to another contractor, Dr. Stanley Nathenson, for the extraction of soluble mouse H-2 antigens.

Major Findings: This contract has supplied 12 additional scientific investigators with approximately 500 ml of mouse H-2 alloantisera for use in studying the immunogenetics of graft rejection and the histocompatibility loci during the past contract year. Similarly, approximately 60,000 mouse spleens have been shipped to Dr. Stanley Nathenson. There seems to be good retention of antibody activity in the banked antisera since no notable differences have occurred after six months evaluation among antisera stored lyophilized, at -20°C or at -60°C. Upon rechecking the specificity of the antisera using a different technique, a number of unpredicted antibodies have now been detected which will modify and improve the original "H-2 Chart" mouse strain combinations needed for making H-2 antisera. These newer combinations are currently being used in producing and resupplying H-2 antisera to the bank.

Significance to NIAID Program and Bio-Medical Research: These reagents are the basic immunogenetic ingredients from which knowledge will be enhanced concerning the immunogenicity and tolerogenicity of the mouse H-2 histocompatibility antigens. This has a direct application to the elucidation of the HLA antigenic system in human transplantation and its role in the rejection phenomenon.

Proposed Course of Project: Continuation through April 30, 1974

Date Contract Initiated: June 1, 1966

Current Annual Level: Prior year support

UNIVERSITY OF LEIDEN (PH43-65-992)

Title: Search for HL-A sera and improvement of histocompatibility techniques

Contractor's Project Director: Dr. Jon J. van Rood

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract seeks to further evaluate the efficacy of the platelet complement fixation, agglutination, and cytotoxic tests for studying histocompatibility antibodies. These techniques are used in selecting donors and recipients in the Eurotransplant Program. This includes transplant units in Holland, Belgium and West Germany that attempts to find the most compatible donor-recipient pairs for transplantation. Organ recipients are then followed to determine the prognostic value of histocompatibility matching. Further, the contract provides funds to search for and collect quantities of antisera containing histocompatibility antibodies. The contractor also is conducting a study in cooperation with the Radiobiological Institute/TNO to compare the histocompatibility antigens in man and a colony of chimpanzees. This contract also provides funds to develop more efficient techniques for reading and recording the results of complement fixation tests for tissue typing.

Major Findings: The contractor has developed a panel of cell donors which are used in a program to find monospecific platelet complement fixation test serum and to discover new specificities. He has deposited 7,124 cc of unique histocompatibility typing serum in the bank during the past fiscal year. Comparisons are being carried out in the Eurotransplant Program to demonstrate the relative efficacy of the complement fixation and the micro-cytotoxicity techniques for histocompatibility typing. Eurotransplant Program recipients of matched cadaver kidneys have had fewer rejection episodes and present less of a management problem than those who receive kidneys with two or more antigens mismatched. Because of the homogenicity of the Netherlands population, the likelihood of achieving a perfect match is greater than in the heterogeneous U.S. population. This makes it possible to achieve more well-matched transplants in Holland. Chimpanzees have been studied and have been shown to contain antigens which cross react with human antiserum. A further elucidation of the antigen similarities between man and the higher ape may lead to new sources of antisera and, under certain circumstances, may facilitate the utilization of subhuman primate organs for transplantation.

Significance to NIAID Program and Bio-Medical Research: This contractor has an excellent facility for obtaining good serum and is the principal source for reagents for the platelet complement fixation tests. This procedure makes it possible to greatly increase the number of typings done by a single technician. The platelets used in the test are readily transportable and survive for long periods of time. The complement fixation test lends itself to automation. The contractors use of photomultiplier tubes to read the results of individual tests afford a rapid and reproducible system for typing. The contractors continued progress will ultimately result in the development of a complete set of reagents for this procedure. His working arrangement with the

Radiobiological Institute/TNO, which has one of the worlds largest colonies of chimpanzee families who have been histocompatibility typed, makes it possible to conduct the comparison between human and subhuman primate histocompatibility antigens.

Proposed Course of Project: Continuation through December 23, 1972

Date Contract Initiated: June 24, 1965

Current Annual Level: $48,650

MEDICAL COLLEGE OF VIRGINIA - VIRGINIA COMMONWEALTH UNIVERSITY (PH43-68-659)

Title: Analysis of antigenic and serologic factors important in human organ transplantation

Contractor's Project Director: Dr. James C. Pierce

Project Officers: Dr. Robert C. Soctt and Mr. C. Eugene Harris

Objectives: This contractor is engaged in the study of renal post-transplant patients to determine whether the presence of histocompatibility antibodies may be used as a means of predicting graft rejection. He is also performing a comparative study of two tissue typing techniques, i.e. the lymphocytotoxicity method of leukocyte matching and the immune adherence method of kidney cell matching, to compare their value in selecting donors and recipients and to determine qualitative antigenic differences in the two types of cells.

Major Findings: The contractor has demonstrated that kidney cell cross-matching can sometimes demonstrate pre-sensitization to a prospective donor in the face of a negative lymphocyte cross-match. He has constructed a model based on the present knowledge of the genetics of the HL-A system, and has demonstrated that his system can account for the observed incidence of hyperacute rejection in recipients of multiple renal transplants. Several patients followed closely after transplant have shown acute rises in histocompatibility antibodies during rejection episodes.

Significance to NIAID Program and Bio-Medical Research: Improvement in the accuracy of histocompatibility typing is essential if the role of the HL-A system in clinical transplantation is to be determined. By detecting immune adherence antibodies, kidney cell cross-matching shows promise as a major improvement in histocompatibility matching. The study of immunological events which occur after transplantation, by means of monitoring the development of HL-A antibodies in post-transplant patients, will be a valuable contribution to the understanding of the rejection process.

Proposed Course of Project: Continuation through December 31, 1972

Date Contract Initiated: January 15, 1968

Current Annual Level: Prior year support ($93,083 for 18 months)

MICROBIOLOGICAL ASSOCIATES, INC. (NIH-71-2443)

Title: Preparation of human histocompatibility typing trays and evaluation of human histocompatibility typing trays and unknown sera.

Contractor's Project Director: Robert-John Trapani, Ph.D.

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contract provides facilities for the production of preloaded histocompatibility typing trays, and for testing of bulk and tray sera.

Major Findings: This contractor has produced 14,000 preloaded histocompatibility typing trays, evaluated 120 unknown sera for antibodies, assessed 20 typing trays containing lyophilized sera, and quality controlled 40 standard typing trays during this contract year. This contractor has increased his standard cell panel from 20 to 55 well characterized cells. During the contract year, this contractor observed and reported that certain batches of plastic trays were defective. This information was disseminated to the U.S. transplant typing facilities as a possible reason for inadequate typing results.

Significance to NIAID Program and Bio-Medical Research: The use of preloaded standard trays containing the best obtainable sera makes it possible for U.S. transplanters to use identical reagents for typing of donors and recipients. This facilitates the use of rare high-quality histocompatibility typing antisera and provides statistically comparable results which will aid in the exchange of organ program as well as the analysis of the importance of histocompatibility matching in organ graft survival. The contract will provide a quality control facility necessary for continued high quality standardization of tissue typing reagents.

Proposed Course of Project: To continue to June 1973

Date Contract Initiated: June 1971

Current Annual Level: $50,000

SCRIPPS CLINIC AND RESEARCH FOUNDATION (NIH-69-2090)

Title: Purified complement components and complement antisera

Contractor's Project Director: Dr. Hans J. Muller-Eberhard

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contract provides selected clinical investigators and medical researchers with highly purified human complement components and their respective antisera; an anaphylatoxin and its inactivator; and a unique complement component which is needed to detect soluble immune complexes.

Major Findings: During the past year this contractor has produced approximately 300 milligrams of complement protein and approximately 700 milliliter of complement component antisera. Over one-half of these products have already been distributed to 55 various clinical investigators to aid in their research on metabolic and graft rejection studies.

Significance to NIAID Program and Bio-Medical Research: Current methods of evaluating homograft rejection are not entirely satisfactory. This contract will aid in establishing another parameter for the clinician to use in accurately assessing or prognosing homograft rejection by *in vitro* tests which denote differential utilization of complement components in transplanted patients.

Proposed Course of Project: Terminated May 22, 1972. This contract was terminated because complementologist are now producing their own reagents and the utilization of these products were not enhancing the area of transplantation as originally anticipated.

Date Contract Initiated: May 23, 1969

Current Annual Level: Prior year support

UNIVERSITY OF WASHINGTON (PH43-67-1435)

Title: Evaluation of tissue typing techniques in clinical homotransplantation

Contractor's Project Director: Dr. E. Donnall Thomas

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor performs leukocyte typing by lymphocytotoxicity and mixed lymphocyte culture for a bone marrow transplant program. He is investigating the genetics of the HL-A antigen system through the typing of large numbers of family members, and is comparing the results of such typing with the clinical outcome of the transplanted marrow. He supplies the Serum Bank with antisera through an active screening program involving multiparous women.

Major Findings: In the last year this contractor has typed 86 potential bone marrow recipients and 320 family members to find that 26 of the patients had eligible sibling donors. In bone marrow transplantation the contractor has found that the only acceptable donors are HL-A matched siblings, defined by lymphocytotoxicity and non-reactivity in mixed lymphocyte culture. Unrelated or less well matched grafts unvariably result in either graft failure or severe graft versus host disease (GVH). However GVH has also been found in the optionally matched sibling pairs indicating that the HL-A system, as currently understood, is not the sole factor determining the histocompatibility of bone marrow transplants. The possible role of neutrophil-specific and other cellular-specific antigen systems is being considered.

Significance to NIAID Program and Bio-Medical Research: Information essential to the determination of the role played by the HL-A system in bone marrow transplantation is being uncovered through the immunogenetic data and clinical followup provided by this contract. In addition the answers to some urgent questions are forthcoming: the importance of mixed lymphocyte reactivity, as a supplement to standard typing, needs to be clarified, as does the significance of neutrophil-specific antigens in clinical transplantation. The procurement of typing serum for the NIAID Serum Bank is necessary for the continued function of the typing reagent program.

Proposed Course of Project: Continuation through December 28, 1972

Date Contract Initiated: June 29, 1967

Current Annual Level: $50,409

BIONETICS RESEARCH LABORATORIES, INC. (PH43-68-1327)

Title: A primate pre-clinical test center for immune study, primarily with ALS

Contractor's Project Director: Dr. Clement C. Darrow

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: The principal objectives in this contract are to maintain a sub-human primate facility for the testing of human antilymphocyte serum (ALS) and to operate an immunology laboratory to conduct a battery of *in vitro* tests. This year, principal emphasis is placed on *in vivo* testing of program-produced rabbit anti-human thymus serum, which is being readied for clinical trials.

Major Findings: During the past year, 29 antilymphocyte preparations were submitted to the contractor for study; these were tested by a battery of *in vitro* techniques and 28 sera were selected for *in vivo* study. Thirty-four Rhesus, and three chimpanzees and six stump tailed monkeys were utilized in this study. The most effective material was rabbit antithymus serum or globulin. Comparisons of the standard three-dose and other ALS regimens were carried out; the three-dose method was shown to be safe and effective, and produced comparable skin graft survival to the longer dosage regimens. Skin graft studies with Rhesus and chimpanzees, using the same ALS for immuno-suppression, showed good correlation of graft prolongation in both species. Cytotoxic titres correlated well with skin graft survival with rabbit but not with horse antihuman thymus serum.

Significance to NIAID Program and Bio-Medical Research: ALS was used for immunosuppression in more than half of the kidney transplants performed during 1971. Few of these sera were of demonstrated efficacy. The only satisfactory means for testing ALS at the present time is the dermal allograft survival test. This contract provides for standardized procedures to conduct comparative testing of ALS preparations. Potency tests and pre-clinical pharmacology studies are also being carried out on a large batch of ALS which has been prepared. With the completion of these studies, this material will be used in a coordinated cooperative clinical evaluation of the efficacy of this immunosuppressive material.

Proposed Course of Project: Continuation through June 27, 1973 with pharmacological studies in Rhesus monkeys and chimpanzees. Additional sub-human primate testing will be utilized during the clinical trial period.

Date Contract Initiated: June 28, 1968

Current Annual Level: $202,400

JOHNS HOPKINS UNIVERSITY (PH43-66-924)

Title: Factors related to survival of bone marrow allografts in man

Contractor's Project Director: Dr. George W. Santos

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract evaluates and correlates methods of histocompatibility testing and immunosuppression with the clinical results of allogenic bone marrow grafts in man and studies the efficacy of cyclophosphamide as an immunosuppressive drug.

Major Findings: The graft vs. host phenomenon presents a major impediment to successful bone marrow transplantation. In the animal model, the contractor has developed a system for the administration of cyclophosphamide as a means of preventing and reversing rejection. Using these techniques, the contractor is now conducting human bone-marrow transplantation. He has transplanted nine patients since 1968. Methods have been developed to permit early detection of graft vs. host disease and new regimens are being studied in animals for the prophylaxis and treatment of this complication.

Significance to NIAID Program and Bio-Medical Research: Patients with far advanced hematological malignancies and those with congenital or acquired marrow deficiency states constitute a potential group for bone marrow transplants. The survivals, to date, have been rare; the development of good methods of immunosuppression and the control of infection should result in a markedly increased success rate. This contract affords a unique opportunity to study immunologic activity of the transplanted marrow cells in the graft vs. host phenomenon and to assay the relative importance of the histocompatibility antigens.

Proposed Course of Project: Funded through December 20, 1972

Date Contract Initiated: June 29, 1966

Current Annual Level: Prior year support

UNIVERSITY OF TEXAS, M.D. ANDERSON HOSPITAL AND TUMOR INSTITUTE (PH43-68-949)

Title: Suppression of immune responses with drugs and biological products; A search for more effective less myelotoxic immunosuppression

Contractor's Project Director: Dr. Evan M. Hersh

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides funds to evaluate new drugs and antilymphocyte serum for their immunosuppressive activity in transplant recipients and cancer patients; the contractor also develops new methodology for the evaluation of immunosuppression and studies the immune state in patients with uremia and a variety of malignant diseases.

Major Findings: The contractor has investigated a number of drugs and antilymphocyte serum which are currently being used in kidney and heart transplant patients. He has developed standardized methods for the measurement of immune responsiveness and has used these techniques to study patients prior to, during and after therapy. A number of new anti-tumor agents are being evaluated.

Significance to NIAID Program and Bio-Medical Research: The information gained concerning the utility of new immunosuppressants has been most helpful in the management of the post-transplant patient. The standardization of the methodology makes it possible for investigators to compare the utility of drugs in producing immune paralysis. The techniques will be most valuable when attempts are made to induce immune tolerance, since the aim of this procedure is to produce specific tolerance to histocompatibility antigens sparing immunologic reactivity to other antigens.

Proposed Course of Project: Funded through December 20, 1972

Date Contract Initiated: June 21, 1968

Current Annual Level: $196,986

AMERICAN FOUNDATION FOR BIOLOGICAL RESEARCH, BIOMEDICAL RESEARCH INSTITUTE (PH43-68-619)

Title: Freeze-drying of typing sera

Contractor's Project Director: Mr. Vernon P. Perry

Project Officers: Mr. Donald B. Hare and Mr. C. Eugene Harris

Objectives: This contractor is to freeze-dry the human histocompatibility typing sera in the NIAID Serum Bank, is to serve as a reference laboratory on certain of the antisera to see if they can withstand freeze-drying and to continue the work on the freeze-drying of lambda quantities of typing sera in the Falcon Tray.

Major Findings: Thirty-five liters of the Bank's inventory have been freeze-dried in one ml quantities during this fiscal year. A twenty-six cell panel has been developed and their antigen profile confirmed by two outside laboratories. They have tested many freeze-dried antisera atainst their frozen counterparts to determine whether or not the freeze-drying had adversely affected the serum. It was discovered that either a solution of 15% Dextrose or 5% Gelatin will cause the one lambda quantities of antisera in the Falcon Tray to adhere to the walls of the well after freeze-drying, even during shipment at ambient temperatures. Additional work still remains to be done which will insure that the freeze-dried antisera-binder combination is as readable and reliable as the results of the frozen antisera.

Significance to NIAID Program and Bio-Medical Research: The freeze-drying of typing sera will eliminate much of the expense and many of the logistical difficulties in the shipping of frozen sera and NIH Typing Trays. The freeze-dried antisera can be stored at -20°C, thus much of the -70°C freezer space can be eliminated with all of its inherent problems and finally the freeze-drying of an antiserum prolongs its shelf life which is very important when reference reagents are stored for long periods of time.

Proposed Course of Project: Continuation through September 30, 1972

Date Contract Initiated: March 1, 1968

Current Annual Level: $62,342

UNIVERSITY OF MINNESOTA (PH43-68-99)

Title: Preservation of multicellular organisms and whole organs in vitro with emphasis on the canine at sub-zero temperatures.

Contractor's Project Director: Dr. Richard C. Lillehei

Project Officers: Dr. Robert C. Scott and Mr. C. Eugene Harris

Objectives: This contractor has been involved in the development of methods to resuscitate kidneys and other organs after freezing and storage at sub-zero temperatures. He is also studying methods of assessing cellular and organ damage caused by low temperatures.

Major Findings: Strict control of freezing and thawing rates has been achieved by the development of methods and equipment to perfuse kidneys with temperature controlled fluorocarbon liquid and to rapidly thaw the organs with microwave energy. It has been found that the levels of two enzymes (SGOT, LDH) released into the effluent perfusate can be used to assay the damage incurred by an organ during the freeze-thaw cycle. They are studying the value of applying the principles of the medical treatment for frostbite to the resuscitation of frozen kidneys. Special techniques of freezing, including the use of hyberbaria along with perfusates such as glycerol, have been in use to decrease damage caused by intra- and extra-vascular ice formation.

Significance to NIAID Program and Bio-Medical Research: The optimal utilization of organs available for transplantation is compromised at present by the inability to preserve donated organs for more than several days. This contractor takes a multidisciplinary approach to this problem utilizing the expertise of physicians, mechanical engineers, cryobiologists, and animal scientists with experience in cell freezing.

Proposed Course of Project: Terminated February 28, 1972

Date Contract Initiated: December 29, 1967

Current Annual Level: Prior year support ($161,799)

SCRIPPS CLINIC AND RESEARCH FOUNDATION (PH43-68-621)

Title: Study of the etiologic events and antigen-antibody systems in human glomerulonephritis and the course of nephritis in transplanted patients.

Contractor's Project Director: Dr. Frank J. Dixon

Project Officers: Dr. John G. Ray, Jr. and Mr. C. Eugene Harris

Objectives: This contractor analyzes human serum and renal tissue samples from transplant patients for various antigen-antibody complexes involved in the genesis of glomerulonephritis and the etiological events which trigger the immune response leading to glomerulonephritis. This contractor has developed a sensitive detection test for serum antiglomerular basement membrane (GBM) antibodies, and to further evaluate these antibodies in antilymphocyte globulin (ALG) preparations currently used in the therapy of transplanted patients. Additionally, the contractor is investigating the possible role of microbial agents in chronic human glomerulonephritis and is studying antigen-antibody complexes in immune complex nephritis.

Major Findings: This contractor has histologically and serologically evaluated 939 specimens from 540 patients in 35 transplantation and related nephrology program centers. Circulating anti-glomerular basement membrane (GBM) antibodies were detected by sensitive indirect immunofluorescence technique. Deposits of immunofluorescent immunoglobulin and/or complement were found on the glomeruli in 65 per cent of the renal tissues studied. This contractor has shown in 3 cases of anti-GBM antibody induced glomerulonephritis that renal transplantation is successful if circulating anti-GBM antibodies are first allowed to disappear.

In a study of 200 cases of immune complex mediated glomerulonephritis, several high titered anti-EBV sera were found and this herpes-like viral disease appears correlative with this type of nephritis. There also appears to be an association with influenza A2 viral infection and Goodpastures Syndrome. These results question the status of graft rejection and have helped define the mechanisms of renal failure in transplanted patients as being a possible recurred nephritis rather than a histoincompatibility graft rejection.

In the evaluation of ALG preparations used in the therapy of transplanted patients, the results indicate that 70 per cent of the ALG preparations produced with lymphocytes derived from solid tumors contain GBM antibodies, while ALG prepared using cultured lymphocytes have not contained these potentially nephrotoxic antibodies.

Significance to NIAID Program and Bio-Medical Research: This significant research has developed evidence relative to the understanding of acute and chronic glomerulonephritis and its occurrence in transplanted kidneys. It has contributed to the elucidation of the rejection process by the addition of another parameter which must be evaluated by the clinician in management of the transplanted patient. Similarly, all ALS preparations should be screened for presence of anti-glomerular basement membrane antibodies.

Proposed Course of Project: Continuation through March 1, 1973

Date Contract Initiated: January 2, 1968

Current Annual Level: Prior year support

DUKE UNIVERSITY (NIH-71-2335)

Title: Partial support for participation in WHO histocompatibility testing workshop

Contractor's Project Director: Dr. D. Bernard Amos

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provided partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically funds were provided to enable the contractor to obtain white cells from three racial isolate groups. The cells were kept viable by liquid nitrogen freezing and transported to the contractor's laboratory where they were tested with a battery of sera selected by the WHO Expert Committee. The results of this testing will be analyzed and will form the basis of a report which will be given at the meeting of the Committee in the summer of 1972. This study was designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching; linkage studies with red cell antigens were carried out. The contractor also hopes to determine if there is any relationship between the presence of certain histocompatibility antigens and susceptibility to tropical diseases.

Major Findings: The study is completed; results are being analyzed.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explanation for rejections which sometimes takes place in transplants where, using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera will lead to an improved success rate for organ transplantation.

Proposed Course of Project: Completed

Date Contract Initiated: May 1, 1971

Current Annual Level: Prior Year Support

UNIVERSITY OF CALIFORNIA, LOS ANGELES (NIH-71-2334)

Title: Partial support for participation in WHO histocompatibility testing workshop

Contractor's Project Director: Dr. Roy Walford

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provided partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically, funds were provided to enable the contractor to obtain white cells from racial isolate groups. The cells kept viable by liquid nitrogen freezing and were transported to the contractor's laboratory where they were tested with a battery of sera selected by the WHO Expert Committee. The results of this testing were analyzed and formed the basis of a report which will be given at the meeting of the Committee in the summer of 1972. This study is designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching, linkage studies with red cell antigens will be carried out.

Major Findings: The study has been completed; results are being analyzed.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explanation for rejection which sometimes takes place in transplants where using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera would lead to an improved success rate for organ transplantation.

Proposed Course of Project: Terminated

Date Contract Initiated: May 1, 1971

Current Annual Level: Prior year support

STANFORD UNIVERSITY (NIH-71-2336)

Title: Partial support for participation in WHO histcompatibility testing workshop

Contractor's Project Director: Dr. Rose Payne

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provided partial support to conduct histocompatibility testing on selected populations as part of the WHO-sponsored Fifth International Conference and Workshop on Histocompatibility. Specifically, funds were provided to enable the contractor to obtain white cells from Chinese-Americans in California and Indians in New Mexico and Arizona. The cells were kept viable by liquid nitrogen freezing and were transported to the contractor's laboratory where they were tested with a battery of sera selected by the WHO Expert Committee. The results of this testing were analyzed and will form the basis of a report which will be given at the summer meeting of the Committee in 1972. This study was designed to search for exotic antigens in geographically and culturally isolated groups. The discovery of these antigens may lead to a better understanding of the inconsistencies of histocompatibility matching, linkage studies with red cell antigens were carried out. The contractor also hoped to determine if there was any relationship between the presence of certain histocompatibility antigens and susceptibility to tropical diseases.

Major Findings: The population studies have been completed; the results are being analyzed.

Significance to NIAID Program and Bio-Medical Research: At the present time there is good evidence that all of the histocompatibility antigens in the two segregant series of the HLA locus have not been discovered. This may be an explantation for the rejection which sometimes takes place in transplants where, using presently available reagents, the donor and the recipient seem to be well matched. The discovery of missing antigens and their antisera would lead to an improved success rate for organ transplantation.

Proposed Course of Project: Terminated

Date Contract Initiated: May 1, 1971

Current Annual Level: Prior Year Support

NAVAL MEDICAL RESEARCH INSTITUTE, NATIONAL NAVAL MEDICAL CENTER Interagency Agreement

Title: Preparation and testing of anti-human thymocyte serum

Contractor's Project Director: Dr. Kenneth W. Sell

Project Officers: Dr. Donald E. Kayhoe and Mr. C. Eugene Harris

Objectives: This contract provides partial support for a program to immunize rabbits with human thymus cells to produce antilymphocyte serum. The product developed, following extensive pre-clinical pharmacology and toxicity studies conducted under the Bionetics Research Laboratories contract (PH 43-68-1327) will be used for a clinical trial.

Major Findings: Optimum methods for the production and testing of rabbit anti-human thymus serum have been developed. Batch production is underway to continue the sub-human primate pharmacology, toxicity and stability studies needed for a DBS investigational new drug application. Production of a 50-patient supply of antiserum has been started.

Significance to NIAID Program and Bio-Medical Research: This facility is producing antilymphocyte sera of demonstrated efficacy in the prolongation of skin grafts in subhuman primates. The material is being produced under strict quality control procedures to assure potency and purity. Program studies in the past have indicated that very few of the currently employed ALS preparations can be demonstrated to have skin graft surviving properties in sub-human primates. Antilymphocyte serum is needed as an additional measure to prevent homograft rejection. This contract will produce an effective ALS which will be used in a coordinated-protocol study to determine the efficacy of ALS in preventing homograft rejection.

Proposed Course of Project: To terminate June 30, 1972

Date Contract Initiated: July 1, 1970

Current Annual Level: $279,288

4. PUBLICATIONS

A

Balner, H., Gabb, B. W., Dersjant, H., van Vreeswijk, W., van Leewen, A., and van Rood, J.J.: Heterologous antisera for human histocompatibility testing. Transplantation Proceedings. 111:2 1088, 1971.

Balner, H., Gabb, B. W., Dersjant, H., van Vreeswijk, W., van Leeuwen, A., and van Rood, J. J.: Leukocyte groups in non-human primates, their relation to histocompatibility and to human HL-A antigens. Academic Press. 97:128, 1971.

Balner, H., van Leeuwen, A., van Vreeswijk, W., Dersjant, H., and van Rood, J.J.: Leukocyte antigens of Rhesus monkeys (RhL-A) and chimpanzees (ChL-A). Similarities with the human HL-A system. Tissue Antigens. 5:1, 229-38, 1971.

Bashirelahi, N., and McKenzie, I.F.C.: Retention of HL-A antigenicity after lyophilization. in press

Beliel, O. M., Mickey, M. R., and Terasaki, P. I.: Comparison of male and female kidney transplant survival rates. Transplantation. in press

Belzer, F. O., Kountz, S. L., and Perkins, H. A.: Red cell cold autoagglutinins as a cause of failure of renal allotransplantation. Transplantation. 11:422, 1971.

Boehmig, J. J., Giles, G. R., Amemiya, H., Wilson, C. B., Coburg, A. J., Genton, E., Bunch, D. L., Dixon, F. J. and Starzl, T. E.: Hyperacute rejection of renal homografts; With particular reference to coagulation changes, humoral antibodies and formed blood elements. Transplantation Proceedings. 111:2, 1105, 1971.

Boxer, L. A., Yokoyama, M., and Lalezari, P.: Isoimmune neonatal neutropenia. Report of a new case. Blood. in press.

Braun, W. E.: Immunologic aspects of renal transplantation. Ibid.

Braun, W. E., Grecek, D. R., and Murphy, J. J.: Histocompatibility testing. Surg. Clinics of North America. in press.

Cheema, A. R., and Hersh, E. M.: Patient survival after chemotherapy and its relationship to *in vitro* lymphocyte blastogenesis. Cancer. 28:851-855, 1971.

Curtis, J. E., and Hersh, E. M.: Cellular immunity in man: Correlation of cell migration inhibition factor formation and delayed hypersensitivity. Cellular Immunology. in press.

Curtis, J. E., and Hersh, E. M.: The human secondary immune response to Keyhole limpet hemocyanin. Clin. Exptl. Immunol. 78:61-69, 1972.

Curtis, J. E., Hersh, E. M., Butler, W. T., and Rossen, R. D.: Antigen dose in the human response; Dose response relationship in the human immune response to Keyhole limpet hemocyanin. J. Lab. Clin. Med. 78:61-69, 1971.

Curtis, J. E., Sharp, J. T., Lidsky, M. D. and Hersh, E. M.: The immune response to rheumatoid arthritis. Archives of Internal Medicine. in press.

Davis, L. E., Hersh, E. M., Curtis, J. E., Lynch, R. E., Ziegler, D. K., Neumann, J. W. and Chin, T. D. K.: Immune status of patients with multiple sclerosis. Analysis of primary and established immune responses in 25 patients. Neurology. in press.

DiPadua, D., McKenzie, I.F.C., and Russell, P.S.: Extraction and purifications of murine cells surface alloantigens. Fed. Proc. 31:738, 1972.

Dixon, F. J., Wilson, C. B., and Marquardt, H.: Experimental immunologic glomerulonephritis. Proc. Int. Sem. Nephritis, 7, 1971.

Dixon, F. J., Wilson, C. B., and Marquardt, H.: Immunology of glomerulonephritis. Progress in Immunology Academic Press, N. Y., 681, 1971.

Douglas, K. S., Perkins, H. A., Cochrum, K. and Kountz, S. L.: Comparison of the HL-A phenotypes of lymphocytes and kidney cells determined by the fluorochromasia cytotoxicity assay. J. Clin. Invest. 50:274, 1971.

Eysevoogel, V. P., van Rood, J. J., du Toit, E. D., and Schellekens, P.Th.A.: Position of a locus determining mixed lymphocyte reaction apart from the known HL-A loci. Europ. J. Immunol. in press.

Fortner, J. G., Shiu, M. H., Balner, H., Wilson, C. B., Sickuk, G., Kawano, N., Holmes, J. T., and Beattie, E. J.: Observations on prolonged immune suppression for liver homografts. Transpl. Proc. 111:1, 383, 1971.

Fotino, M., Merson, E. J., and Allen, F. H., Jr.: Micromethod for rapid separation of lymphocytes from peripheral blood. Vox Sang.

Freudenberg, J. and van Rood, J. J.: A first report of the Central Registy of Matches. Transp. Proc. in press.

Gibofsky, A., Terasaki, P. I.: Trypsinization of lymphocytes for HL-A typing. Transplantation. 13:192, 1972.

Halgrimson, C. G., Wilson, C. B., Dixon, F. J., Penn, I., Anderson, J. T., Ogden, D. A., and Starzl, T. E.: Goodpasture's syndrome: Treatment with nephrectomy and renal transplantation. Arch. Surg. 103:283, 1971.

Hersh, E. M.: Basic considerations: Inhibition of antibody synthesis, suppression of delayed hypersensitivity and drug induced immunologic tolerance. Handbuch der Experimental Parmacologie. Springer-Verlag, Berlin, Heidelberg, New York. in press.

Hersh, E. M.: Modification of host-defense mechanisms by chemotherapy. Cancer Medicine. Lea & Febiger, Phila. Penna. in press.

Hersh, E. M.: Immunosuppression by L-asparaginase and related enzymes: A review. Transplantation. 12:368-376, 1971.

Hersh, E. M.: Blastogenic responses of human lymphocytes to xenogeneic cells in vitro. Transplantation. 12:287-293, 1971.

Hersh, E. M., Butler, W. T., Rossen, R. D., Morgen, R. O. and Suki, W.: In vitro studies of the human responses to organ allografts; appearance and detection of circulating activated lymphocytes. J. Immunol. 107:571-578, 1971.

Hersh, E. M. and LePage, G. A.: The effect of arabinosyl-6-mercaptopurine on the in vitro blastogenic responses of human lymphocytes to mitogenic agents. Biochem. Pharmacol. 20:2459-2468, 1971.

Hersh, E. M., Whitecar, J. P., Jr., McCredie, K. B., Bodey, G. P., Sr. and Freireich, E. J.: Chemotherapy, immunocompetence, immunosuppression and prognosis in acute leukemia. New Engl. J. Med. 285:1211-1216, 1971.

Hume, D. M., Lee, H. M., Pierce, J. C., and Cobb, G. W.: Histocompatibility concomitants of the clinical course of renal homografts. Transplantation Proceedings 3:371-375, 1971.

Jeekel, J., McKenzie, I.F.C., and Winn, H. J.: Skin graft enhancement in the mouse. J. Immunol. in press.

Koch, C. T., Frederiks, E., Eysvoogel, V. P. and van Rood, J. J.: Mixed lymphocyte culture and skin graft data in unrelated HL-A identical individuals. Lancet. II:7738, 1334-1336, 1971.

Kux, M., Boehmig, H. J., Amemiya, H., Torisu, M., Yokoyama, T., Launois, B., Popovtzer, M., Wilson, C. B., Dixon, F. J. and Starzl, T. E.: Modification of hyperacute canine renal homograft and pig to dog heterograft rejection by the intraarterial infusion of citrate. Surgery. 70:103, 1971.

LePage, G. A. and Hersh, E. M.: Cyclic nucleotide analogs as carcinostatic agents. Biochem. Biophys. Res. 46:1918-1922, 1972.

Lalezari, P., Murphy, G., and Allen, F. H., Jr. NB1, a nutrophile-specific antigen involved in the pathogenesis of neonatal neutropenia. Journal of Clinical Investigation. 1971.

McKenzie, I. F. C. and DiPadua, D.: Extraction of H-2 antigens with detergents. in press.

McKenzie, I. F. C., and DiPadua, D.: Solubilization of H-2 antigens using 3M KCL. in press.

McKenzie, I. F. C., Painter, E.: The antibody response of rabbits to soluble mouse H-2 antigen. J. Immuno. in press.

McKenzie, I. F. C., Painter, E., Winn, H. J., Antibody production in rabbits to soluble H-2 antigens. Fed. Proc. 30:523, 1971.

Merkel, F. K., Bier, M., Beavers, C. D., Merriman, W. G., Wilson, C. B., and Starzl, T.E.: Modification of the xenograft response by selective plasmapheresis. Transpl. Proc. III:1, 534, 1971.

Mickey, M. R., Kreisler, M., Albert, E. D., Tanaka, N., and Terasaki, P. I.: Analysis of HL-A incompatibility in human renal transplants. Tissue Antigens 2:57, 1971.

Mittal, K. K., and Terasaki, P. I.: Cross-reactivity in the HL-A system. Tissue Antigens. in press.

Mittal, K. K., Mickey, M. R., Terasaki, P. I.: Heterogenicity of HL-A specificities shown by platelet complement fixation. Tissue Antigens. 1:279, 1971.

Opelz, G., Mickey, M. R., Terasaki, P. I.: Identification of unresponsive kidney transplant recipients. The Lancet. in press.

Payne, R., Perkins, H. A., Kountz, S. L. and Belzer, F. O.: Unrelated kidney transplants and matching for HL-A Antigens. Transplant. Proc. 3:1036, 1971.

Perry, V. P., Kroener, C.: The design of a chamber for freeze drying bulk microtiter quantities of tissue typing serum. in press.

Perry, V. P., Kroener, C.: The use of binders for the retention of microtiter quantities of serum in tissue typing trays. in press.

Pierce, J. C., Cobb, G. W., Hume, D. M.: Relevance of HL-A antigens to acute humoral rejection of multiple renal allotransplants. N.E.J.M.285 142-146, 1971.

Pierce, J. C. and Hume, D. M.: Cross matching for organ transplantation I. The use of kidney cells and immune adherence. Transplantation Proceedings 3:127-129, 1971

Pierce, J. C., Madge, G. E., Lee, H. M., and Hume, D. M.: Lymphoma, A complication of renal allotransplantation in man. JAMA. in press.

Rosenberg, E., Mann, D. L., Hill, J. L., Fahey, J. L. and Nathenson, S. G., Transplantation. 12:402, 1971.

Salmon, S. E., Mogerman, S. N., Perkins, H. A., Smith, B. A., Lehrer, R. I., and Shinefield, H. R.: Transplantation of treated lymphocytes in lymphopenic immunologic deficiency. in press.

Salmon, Se. E., Schrier, R. W., and Smith, L. H. Jr.: Immunological mechanisms of glomerulonephritis. California Medicine. 116:47-52, 1972.

Sengar, D. P. S., Mickey, M. R., Myhre, B. A., Chen, G. H., and Terasaki, P. I.: Mixed leukocyte culture response in HL-A "Identical Individuals." Transfusion. 11(15):251, 1971.

Sengar, D. P. S., and Terasaki, P. I.: A semi-micro mixed leukocyte culture test. Transplantation. 11(3):260, 1971.

Tanaka, N., Stevens, L. E., and Terasaki, P. I.: Storage and transport of 83 human kidneys by simple hypothermia. Transplantation. 12:348, 1971.

Tanaka, N., Takasugi, M., and Terasaki, P. I.: Presensitization to transplants detected by cellular immunity tests. Transplantation. 12:514, 1971.

Terasaki, P. I., and Mickey, M. R.: Histocompatibility transplant correlation, reproducibility and new matching methods. Transplantation Proc. 3:1057, 1971.

Terasaki, P. I., Mickey, M. R., and Kreisler, M.: Presensitization and kidney transplant failures. Postgrad. Med. J. 47:89, 1971.

Terasaki, P. I., Wilkinson, G., McClelland, J.: National Communications Network. J. Amer. Med. Assoc. 218:1674, 1971.

van Rood, J. J.: The (relative) importance of HL-A matching kidney transplantation. Acad. Press, Progress in Immunol. 1027-1043, 1971.

van Rood, J. J., Freudenberg, J., van Leeuwen, A., Schipper, H. M. A., Zweerus, R. and Terpstra, J. L.: Eurotransplant. Transpl. Proc. III:1, 933, 1971.

van Rood, J. J., and van Leeuwen, A.: HL-A and group five system in Hodgkin's disease. Transpl. Proc. III:3, 1283-1286, 1971.

van Rood, J. J., van Leeuwen, A., and Balmer, H.: HL-A and ChL-A. Similarities and differences. Transp. Proc. IV:1, 55-62, 1972.

van Rood, J. J., van Leeuwen, A., de Graaf, S., and Mowbray, J.: Relationship between kidney graft prognosis and HL-A typing using a micro-absorption technique. Transpl. Proc. III:2, 1051-1056, 1971.

van Rood, J. J., van Leeuwen, A., Freudenberg, J. and Schippers, H. M. A.: Gewebstypisierung. Grundlage fur die klinische Organtransplantation. Langenbecks Archiv (Kongress 1971) Springer-Verlag, Heidelberg, 1971.

van Rood, J. J., van Leeuwen, A., Freudenberg, J. and Rubinstein, P.: Prospects in host-donor matching. Transpl. Proc. III:2, 1042-1050, 1971.

Weerdt, Ch. van der, and Lalezari, P.: Another example of isoimmune neonatal neutropenia due to an-NA1. Vox Sanguinis. in press

Wilson, C. B., Immunologic mechanisms of glomerulonephritis. Calif. Med. in press.

Wilson, C. B. and Dixon, F. J.: The pathogenesis of glomerulonephritis. Textbook of Internal Medicine. in press.

Wilson, C. B., and Dixon, F. J. Immunological aspects of glomerulonephritis. Textbook of Renal Disease. in press.

Wilson C. B. and Dixon, F. J.: Quantitation of acute and chronic serum sickness in the rabbit. J. Exp. Med. 134:7, 1971.

Wilson, C. B., Dixon, F. J., Fortner, J. G., and Cerilli, G. J.: Glomerular basement membrane reactive antibody in anti-lymphocyte globulin. J. Clin. Invest. 50:1525, 1971

Wilson, C. B. and Smith, R. C.: Goodpasture's syndrome associated with influenza A2 virus infection. Ann. Int. Med. 76:1, 91-94, 1972.

B - Staff Publications

Hare, D. B., Harris, C. E., Kayhoe, D. E., and Ray, J. G. Jr., (Eds.): Catalog of Tissue Typing Antisera, 1971. U. S. Dept. of Health, Eduation, and Welfare, National Institutes of Health, Bethesda, Maryland, 1971, 803 pp.

Kayhoe, D. E.: The Evaluation of the results of histocompatibility typing. The American Journal of the Medical Sciences. in press.

Kayhoe, D. E., and Hare, D. B.: A Histocompatibility Typing Serum Bank. Transplantation Proceedings. 2: 988-992, 1971.

Ray, J. G. Jr., Hare, D. B., and Kayhoe, D. E., (Eds): Transplantation and Immunology Catalog Supplement, May 1971. U. S. Dept. of Health, Education and Welfare, National Institutes of Health, Bethesda, Maryland, 1971, 30 pp.

RESEARCH RESOURCES BRANCH

1. Summary

The Research Resources Branch provides well characterized reagents to the research community in cases where reagents are not readily available from other sources. These reagents for health research purposes include seed viruses and mycoplasma and corresponding antisera characterized by immunological and biochemical methods.

The production of seed and antisera to the most important viruses and mycoplasma needed in health research is complete. As needed, the program sponsors the remaking of reagents which had either dropped in titer or were not of the highest quality from the start. Enterovirus identification pools are now ready for distribution. Arbovirus grouping pools have also been prepared and pools for rhinovirus identification will be initiated when the next phase of production is complete. Prospective new reagents include antisera to influenza virus neuraminidase and hemagglutinin. Mycoplasma reagent production has been completed and certification of reagents should be completed in the near future.

The arbovirus reagent program is entering into its final phase of production activity. Five arbovirus production contracts have been terminated and one other will be terminated at expiration since program goals should be realized in a year or two. Two dengue arboviruses have been assigned to a contractor since suitable immunization schedules for the production of specific reagents have been developed. Three mosquito blood meal antisera are being produced. This will complete the program goal of producing 12 mosquito blood meal antisera.

The project to produce interim reagents for hepatitis B antigen (HB Ag), subtype ad, was successfully completed in July 1970 through the combined efforts of Intramural Scientists, the NIAID/AEC MAN Laboratory and the CDC. These reagents have been highly utilized and program is presently having produced new reagents representing the ay antigenic subtype. The program has sponsored working conferences designed to obtain advice and guidance on new projects for producing hepatitis reagents.

Antigen E prepared from dwarf ragweed pollen is packaged and available for distribution. Antigen Ra 3 is currently in production and proposals have been solicited for future production of additional reagents to antigen E, K, Ra 2, 3, 4, and 5. The cytomegalovirus reagents have been produced, and are currently being tested.

The rhinovirus reagent program for the Phase I rhinoviruses (1-55) is nearing completion with many certified seed virus reagents and sera available for distribution. The production of Phase II rhinovirus types (56-89) has commenced and these reagents should be available by late 1972.

With the publication and distribution of the 1970 Research Reagents Catalog

there has been a continuing publicizing of the availability of new reagents and reference to the 1970 Catalog is reaching investigators not previously aware of reagent availability. A supplement to the 1970 catalog will be published in the near future.

The program derives continued support from the NIH Intramural Laboratories which furnish scientific and technical consultation, project officers, and ad hoc Committee members for review of new and ongoing programs.

2. Program Status Report

Adenoviruses

Remake of adenovirus seed which had dropped in titer and antisera which demonstrated low or excessive cross reactivity is almost completed. It is estimated that the new seeds for Types 8, 9, 13, 15, 16, 17, 18, 22, 23, 24, 26 and 27 will be packaged and ready by September 1972 and that the new antisera to Types 2, 4, 8, 9, 10, 13 and 14 will be packaged and ready before the end of 1972.

Arboviruses

Three contractors are actively engaged in the production of reference reagents to nine arboviruses. The certification laboratory has tested one hundred and ten arbovirus reference reagents which were subsequently recommended for distribution by the Arboviruses Committee. Forty-eight bulk reagents are in the process of being tested prior to final processing.

A contract for the production of antibody-containing grouping ascitic fluid has resulted in the production and approval for distribution of seventeen grouping fluids. Thirteen others are in various stages of testing prior to final processing.

Current plans call for the remaking of three mosquito blood meal antisera. These antisera will contain antibody against the blood of anti-ciconiiform, anti-columbiform, and anti-passeriform birds. When these are complete there will be a total of 12 antisera available for distribution. There are four major animal groups and nine are for the most useful representatives of avain, mammalian and reptilian species.

Table I and 2 summarize the production status of arbovirus reagents.

Myxoviruses

With new techniques available the Staff is carrying out plans to produce antisera to all 23 of the known neuraminadases and hemagglutinins of influenza viruses of man and animals. A number of these have already been produced in bulk and will soon be packaged. The Staff is also sponsoring the production, testing and evaluation of a small lot of 3' methoxphenyl-N-α-neuramic acid (MPN) which should provide useful in demonstration of neuraminadase and will also have application in interferon assays involving influenza viruses.

The Branch sponsored a workshop on the parainfluenza viruses in November 1971. The workshop brought together scientists from medical and veterinary disciplines. There were useful discussions of paramyxoviruses of man and animals and the group identified areas of research needed to better classify the paramyxoviruses. A summary of the meeting has been submitted to the Journal of Infectious Diseases and the full report will be submitted to the WHO Bulletin.

The production of seed virus and antisera for myxovirus reagents has been completed.

Enteroviruses

The enterovirus typing pools Lim Benyesh-Melnick have been completed, packaged and evaluated and are now available for distribution. It is anticipated that these pools will contribute to the rapid identification of enteroviruses and to the recognition of new types.

Rhinoviruses

Phase I of the rhinovirus reagents program to prepare seed virus and guinea pig antisera to fifty-six prototype rhinoviruses is nearing completion. At this time there are forty-six final vial seed viruses that have been approved for distribution as reference reagents; five have been rejected and five are currently being distributed as interim reagents. Fifty-four final vial antisera are being tested and two bulk antisera have been rejected because of low titer and are being remade.

Sufficient guinea pig antisera and certified seed has been prepared by the contributors involved in the Phase II of the characterization program and adequate authenticating serum and seed has been made available to five contractors currently producing thirty-four Phase II rhinoviruses and any remakes of the unacceptable Phase I reagents.

The number of accepted rhinovirus prototypes, representing Phase I and Phase II now totals eighty-nine.

Other Viruses

AAV

The Branch is sponsoring production of the following AAV types 1, 2, 3, and 4. It is anticipated that these will be available by the end of 1972.

Cytomegaloviruses

Production of cytomegaloviruses has recently been completed and samples of final packaged seed and bulk sera are currently being tested by the certification laboratory.

Coronaviruses

The program has initiated the production of two types of coronaviruses (OC-43 and 229E). The production of these reagents should be complete by June 30, 1972. The RRB sponsored a workshop on the coronaviruses which brought together medical and veterinary science from three foreign countries. The workshop was very useful in identifying additional work which was needed to relate some of the coronaviruses of man and animals. A summary of the meeting has been submitted to the Journal of Infectious Diseases and the full report will be submitted to the WHO Bulletin.

Mycoplasma

The production of mycoplasma reagents is now completed. This has been a closely coordinated program between the Research Resources Branch, NIAID and the Division of Biologics Standards for the preparation of reference reagents (seed, corresponding mule or donkey antisera) and conjugated serum to twenty-eight mycoplasma species of human and non-human origin. The final report has been received from the certification laboratory (University of Aarhus). The results are now being analyzed. The information contained in the report is very detailed and includes homologous and heterologous test results against a large battery of reagents. Twenty-five seed reagents, seven final ampouled antisera, twenty-one minipool and twenty-eight conjugated antisera have been tested by the certification laboratory. The data obtained are comparable with the results obtained by the producer and the Laboratory of Infectious Diseases.

AEC-NIAID Molecular Anatomy Program (MAN)

The Interagency Program is serving to develop methodology for the analysis for viral antigens and antibody with research conducted at the Molecular Anatomy (MAN) Program in Rockville, Maryland. The Staff of the MAN Program has been working in close collaboration with Intramural NIAID Scientists on projects involving respiratory and hepatitis viruses. These programs encompass the biophysical and biochemical characterization of respiratory syncytial virus and its temperature sensitive mutants, the concentration and purification of parainfluenza and recombinant influenza viruses, and various studies with the HB Ag.

The MAN Program laboratory has been increasingly concerned with the immunochemistry of HB Ag and has produced useful reagents for HB Ag subtypes. Five combinant influenza vaccines have been prepared which are either in or scheduled for clinical studies.

Antigen E

Purified antigen E fractionated from ragweed pollen, is now being distributed by the Branch for both in-vivo and in-vitro research.

The Branch is now developing plans to award contracts for the production of additional antigen E and new reagents for antigens K, and Ra 3. The Advisory Committee recommended that reagents be prepared for Ascaris lumbricoides and allergens for rye grass.

Hepatitis

The interim reagents for hepatitis B antigen, first made available in 1970, continue to be in demand and have proven of considerable value. Recent studies on subtypes of HB Ag have revealed that the NIAID interim reagents were of subtype ad. Therefore, additional reagents are being produced to the other major subtype ay.

It is hoped that through intensive evaluation of the ad and ay reagent antisera,

these materials will be accepted as WHO International Reference Standards. The subject of reagents and nomenclature were discussed at some length at the Second Workshop on Antigenic Subtypes of HBAg sponsored by the Research Resources Branch on December 17, 1971. There was general agreement on use of the term of hepatitis B antigen (HBAg) in describing what has been previously called Australia (Au) antigen, Hepatitis Associated antigen (HAA) or Serum Hepatitis (SH) Antigen. The antibody for HBAg can best be termed anti-HBAg or HBAb. The causative viruses of hepatitis type A and hepatitis type B, if and when found, should be called hepatitis A virus (HAV) and hepatitis B virus (HBV) respectively. Subtypes of HBAg should retain the descriptors ay and ad for the time being.

At a Collaborative Research sponsored conference on Immune Complexes and Hepatitis produced a variety of recommendations on new hepatitis reagents including rheumatoid factor and Clq. It was also apparent that reagents for the inner core of the "Dane particle" were urgently needed.

Distribution of Reagents

Fiscal Year	Period of Operation	Total Transactions	Total Amps & Vials Distributed
1968	7/1/67-6/30/68	391	31,776
1969	7/1/68-6/30/69	402	15,805
1970	7/1/69-6/30/70	474	26,242
1971	7/1/70-6/30/71	638	12,902
1972	7/1/71-3/31/72 (9 months)	399	14,960

A total of 65,974 ml of bulk has been distributed since the program was initiated.

The number of transactions to date is slightly lower than that of last year this time. However, the total number of ampoules or vials is greater than the total for last year. This increase can be attributed to a large number of reagents being transferred to the Division of Biologics Standards and to the Center for Disease Control. The total number of shipments for 1971 included 175 shipments of Australia (Hepatitis-Associated) antigen reagents. It is expected that an increase of the number of shipments will occur in FY 1973 when the Australia (Hepatitis-Associated) reagents for subtype ay become available sometime during the summer.

The 1970 Catalog of Research Reagents was completed in November 1970. The Catalog has been sent to approximately 1500 investigators. The Catalog listed and described over 100 new reagents including mycoplasma reagents which had not appeared in the 1968 catalog. Work has now been initiated on a supplement to the 1970 Catalog. The supplement will list and describe guinea pig reference antisera types 1 through 55, all the mycoplasma reagents and additional arbovirus reagents.

TABLE I

Arbovirus Production and Testing of Arbovirus Reagents to Date

Approved for Distribution

Seed Virus Reagents

Anopheles A	EEE	Modoc	SLE
Anopheles B	EHD	Naples SFF	Tacaribe
Bebaru	Getah	Nepuyo	Tembusu
Bimiti	Guama	Oriboca	Tensaw
Bluetongue	Guaroa	Oropouche	Trivitattus
Buttonwillow	Hart Park	Patois	VEE
Bwamba	Highlands J	Powassan	VSV (Ind.)
California Enceph.	Hughes	Rio Bravo	VSV (N.J.)
Candiru	Ilheus	Ross River	WEE
Caraparu	Kern Canyon	Sawgrass	WN
Catu	Lone Star	Semliki Forest	Wyeomyia
Chagres	Manzanilla	Sicilian SFF	YF
Changuinola	Mayaro	Silverwater	Zegla
Colorado TF	MML	Sindbis	

Final Ampoule Being Tested

Cowbone Ridge
JBE
Main Drain
Melao
Punta Toro

Bulk Reagent Being Tested

Tamiami
Junin
Congo

TABLE 2

Arbovirus Production and Testing of Arbovirus Reagents to Date

Approved for Distribution

Immune Ascitic Fluid

Anopheles A
Anopheles B
Buttonwillow
Bwamba
California Enceph
Candiru
Catu
Chagres
Colorado TF
EEE
Group A
Group B
MML
Modoc
Oropouche
Patois
Rio Bravo
Naples
Group C
Group Bunyamwera
Group Capim
Group California
Polyvalent Polyam
Polyvalent Anopheles A
Polyvalent Bwamba
Group Guama
Group Quarafil
Group Simbu
Group Tacaribe
Group VSV
Sicilian SFF
Silverwater
Turlock
VEE
VSV (Ind)
Polyvalent 8
Polyvalent 2
Polyvalent 6
Guama
Guaroa
Hart Park
Hughes
Ilheus
Kern Canyon
Manzanilla
Mayaro
Melao
VSV (NJ)
WN
Wyeomyia
YF
Zegla

Final Ampoule Being Tested

Changuinola
Cowbone Ridge
EHD (NJ)
Nepuyo
Turlock
VEE

Bulk Reagent Being Tested

Bebaru
Bimiti
Chikungunya
Cocal
Congo
Getah
Highlands J
Junin
Lone Star
Main Drain
Microtus
Nepuyo
Nepuyo
Oriboca
Punta Toro
Sawgrass
SLE
Sindbis
Tacaribe
Tamiami
Tensaw
Trivitattus
WEE

3. Contract Narratives

Arbovirus Reagent Program

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (PH43-68-679)

Title: Production and Testing of Arbovirus Research Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officers: Dr. Karl M. Johnson and Mr. Robert M. Pennington

Objectives: To produce research reference reagents to St. Louis Encephalitis, Rio Bravo, Yellow Fever (17D), Tembusu, WN, Bussuquara, RSSE, JBE, Junin, Powasson and Semliki Forest.

Major Findings: In the past year production and testing have been completed on SLE, Rio Bravo, YF, Tembusu, WN, Bussuquara, Junin and Powasson. Production has recently been initiated on RSSE and Semliki Forest.

Significance to NIAID Program and Bio-Medical Research: This contract will furnish ten sets of arbovirus reagents to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: This project has been renewed for an additional and probably final year as part of a continuing arbovirus reagent production effort.

Date Contract Initiated: April 22, 1968

Current Annual Level: $56,358

CENTER FOR DISEASE CONTROL

Title: Catalogue of Arthropod-borne Viruses of the World

Project Director: Dr. Trygve O. Berge

Project Officers: Dr. Earl Chamberlayne and Mr. Robert M. Pennington

Objectives: To maintain a registry of data derived from investigators on arthropod-borne viruses by cataloging the known physical, chemical and antigenic characteristics, the host and vector range, pathogenesis and geographical distribution of these reagents.

Major Findings: To date six new catalogue sets have been issued and the active list of participants include 145 individuals or organizations. Distribution of the Catalogue materials involved 61 mailings within the continental USA and 84 to foreign addresses in 48 countries. Distribution by continent was as follows: Africa 12; Australia (including Hawaii) 8; Asia 19; Europe 26; North America 66; and South America 14.

Significance to NIAID Program and Bio-Medical Research: The continued dissemination of the scientific information pertaining to arboviruses is an essential adjunct to research in arbovirology.

Proposed Course of Project: It is anticipated that this Interagency Agreement will be continued in FY 1973.

Date Contract Initiated: October 1971

Current Annual Level: $20,410 (9 months)

UNIVERSITY OF WEST INDIES (PH43-67-65)

Title: Production and Testing of Arbovirus Reference Reagents

Project Director: Dr. Pierre Ardoin

Project Officers: Dr. Karl M. Johnson and Mr. Robert M. Pennington

Objectives: The reagents assigned to the University for production are those arboviruses indigenous to the area or originally isolated in Trinidad and considered to be a potential health hazard to the U. S. mainland.

To produce and test reference reagents for Oropouche, Mayaro, Ilheus, Melao, Guama, Oriboca, Caraparu, Wyeomyia, Nepuyo, Bimiti, Cocal, Catu, Tacaribe, Gumbo Limbo, Lokern, Bunyamwera and Piry.

Major Findings: The following reagents have been produced and certified for distribution: Oropouche, Mayaro, Ilheus, Melao, Guama, Nepuyo, Bimiti, Wyeomyia, Oriboca and Catu.

In the past year Oriboca, Cocal, Tacaribe, and Caraparu have been produced and are currently being tested by the certification laboratory. Production of Gumbo Limbo and Lokern has been completed and production of Bunyamwera and Pixuna was recently initiated.

Significance to NIAID Program and Bio-Medical Research: This project will furnish seventeen arbovirus reference reagent sets to help fill the needs of NIH grantees, intramural scientists, contractors and other eligible recipients of arbovirus reagents.

Proposed Course of Project: To be terminated in July 1972.

Date Contract Initiated: February 1967

Current Annual Level: Prior year support

YALE ARBOVIRUS RESEARCH UNIT (NIH-72-2509)

Title: The Production of Specific Dengue Immune Reagents

Project Director: Dr. Robert Shope

Project Officer: Mr. Robert M. Pennington

Objectives: To carry out a specific immunization schedules to produce and test dengue immune ascitic fluids for dengue types 2 and 3.

Major Findings: None. Project recently funded.

Significance to NIAID Program and Bio-Medical Research: It is hoped that the immunization schedule developed at Walter Reed Army Institute of Research will result in the production of specific immune reagents to dengue 2 and 3.

Proposed Course of Project: Dependent upon outcome of present production effort.

Date Contract Initiated: February 1972

Current Annual Level: $18,950

YALE ARBOVIRUS RESEARCH UNIT (PH43-66-460)

Title: Testing and Identification of Arbovirus Reagents

Project Director: Dr. Robert Shope

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To furnish essential testing and certification services for other arbovirus production contractors.

Major Findings: To date these testing services have been utilized to furnish NIAID Staff and Arbovirus Advisory Committee with information needed to determine acceptability for distribution of seventy-six arbovirus reagents. Also, serological testing necessary for approval of the next step in processing of bulk reagents has been completed or initiated for thirty-nine arbovirus reagents. In the past year seventy-six reagents were tested and approved for distribution of processing.

Significance to NIAID Program and Bio-Medical Research: This service is an essential element of the arbovirus reagent program and it is anticipated that its full usefulness is now being realized.

Proposed Course of Project: Continuation during arbovirus production and processing efforts.

Date Contract Initiated: January 19, 1966

Current Annual Level: $89,480

FLOW LABORATORIES (NIH-71-2288)

Title: Production of Adenovirus Reagents

Project Director: Dr. Jerome Kern

Project Officers: Dr. Malcolm Hoggan and Mr. Robert M. Pennington

Objectives: To produce, test and package AAV free seed virus reagents to twelve adenovirus types and antisera to seven adenovirus types.

Major Findings: Adenovirus seed virus reagents types 9, 13, 16 and 17 have been approved for packaging and the remaining seed and antisera are currently in production.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus and myxovirus group, with cold like respiratory diseases.

Proposed Course of Project: To be extended to October 1972.

Date Contract Initiated: June 1971

Current Annual Level: Prior year support

CENTER FOR DISEASE CONTROL

Title: Certification of Adenovirus Reagents

Project Director: Dr. Walter Dowdle

Project Officer: Mr. Robert M. Pennington

Objectives: Perform laboratory tests required to certify the seed viruses to twelve adenovirus types and antisera to seven adenovirus types.

Major Findings: Adenovirus seed virus reagents types 9, 13, 16 and 17 have been approved for packaging.

Significance to NIAID Program and Biomedical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the rhinovirus group, with cold like respiratory diseases.

Proposed Course of Project: To be extended to December 1972

Date Contract Initiated: July 1971

Current Annual Level: $24,840

NORTH AMERICAN BIOLOGICALS, INC. (NIH-71-2100)

Title: Production of AAV Reagents

Project Director: Dr. Robert R. Rafajko

Project Officers: Dr. James Rose and Mr. Robert M. Pennington

Objectives: To produce, test and package seed virus reagent and antisera to four adeno-associated virus reagents.

Major Findings: Bulk antisera for two of the four AAV reagents is currently in production.

Significance to NIAID Program and Bio-Medical Research: This contract will furnish four adeno-associated virus reagents which are an essential part of the total adenovirus reagent program due to their adventitious nature in adenovirus seeds.

Proposed Course of Project: To be terminated June 1972

Date Contract Initiated: April 6, 1971

Current Annual Level: Prior year support

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-69-89)

Title: Certification Testing of Enterovirus Reference Reagents

Project Director: Dr. Edwin H. Lennette

Project Officers: Dr. Robert J. Byrne and Mr. Robert M. Pennington

Objectives: To perform certification testing of seed virus and antiserum prepared to prototype enteroviruses. In addition, testing of other virus groups will be performed on request.

Major Findings: The certification laboratory has confirmed the test data as given by the producer or in some cases there have been indications of loss of titer and in several cases contamination with other viruses have been determined. The contractor has completed the certification testing of both seed and monkey antisera prepared for enteroviruses and has completed testing the final ampoules of the LMB pools. The contractor has tested echovirus type 8 and 18 immune horse sera; performed neutralization breakthrough tests on three enterovirus preparations; cross neutralization tests on Toluca 1; and cross neutralization testing of three bulk cytomegalovirus strains.

Significance to NIAID Program and Bio-Medical Research: The contractor provides data confirming authentication, identification, potency and purity of enterovirus reference reagents, produced by independent contractors. This comprehensive certification testing enhances the value of the reagents as reference standards.

Proposed Course of Project: As long as a contract is in effect for the preparation of enterovirus reference reagents the services of this laboratory will be needed. The CSDPH and Center for Disease Control will handle future enterovirus program activities for the Research Resources Branch.

Date Contract Initiated: December 11, 1971

Current Annual Level: $28,535

CENTER FOR DISEASE CONTROL

Title: Produce seed virus reagents for two Picornaviruses

Project Director: Dr. Morris T. Suggs

Project Officer: Mr. Robert M. Pennington

Objectives: Production of the following picornavirus research reference reagents; Coxsackie A-17 and Enterovirus 68 (Fermon).

Major Findings: Master seed virus has been prepared for Coxsackie A-17 and Enterovirus 68 is currently in production.

Significance to NIAID Program and Biomedical Research: The production of these reagents will make available standard reference reagents which are an essential part of those picornaviruses presently in the Research Resources Branch repository.

Proposed Course of Project: Will be terminated June 1972

Date Contract Initiated: July 1971

Current Annual Level: $15,600

UNIVERSITY OF AARHUS (PH43-67-682)

Title: To authenticate and Certify Mycoplasma Reference Reagents Prepared to Human and Other Mycoplasma Strains

Project Director: Dr. E. A. Freundt

Project Officers: Dr. Joseph G. Tully and Mrs. Sylvia Cunningham

Objectives: To certify mycoplasma reference reagents prepared to mycoplasma species for identity, potency and purity. Purity testing will be performed with a known spectrum of mycoplasma species including those which are not available in the United States because of importation regulations. In addition, the Contractor will develop new techniques and/or modify or evaluate existing techniques for the detection, identification or characterization of mycoplasma species.

Major Findings: The contractor has completed the testing of all mycoplasma miniature serum proportionate pools (minipools) for homologous titers by DI and IHA. In addition to the homologous testing the contractor has conducted heterologous testing on the minipools. Twenty-eight conjugated antisera have been tested for homologous titers and for cross-reactivity with three "exotic" strains by direct immunofluorescence on agar; seed reagents have been tested for viable counts and have been tested biochemically and serologically against homologous and heterologous antisera.

In addition, the University of Aarhus has produced small batches of mycoplasma seed and antiserum reagents (against some 40 strains) as needed to perform the above testings.

Production, sterility and safety testing of the equine antisera to three exotic mycoplasma species was completed. These antisera have now been tested at Plum Island and are now available for distribution.

Significance to NIAID Program and Bio-Medical Research: Certification of mycoplasma reagents by the University of Aarhus is essential as a safeguard in insuring authenticity and purity. Specialized testing by the University of Aarhus, for which this laboratory is uniquely qualified, will further enhance the value of the reagents as reference standards.

Proposed Course of Project: Contract is completed. Final report will be received shortly.

Date Contract Initiated: April 28, 1967

Current Annual Level: Prior year support.

CENTER FOR DISEASE CONTROL

Title: Production of Coronavirus Research Reagents

Project Director: Dr. Morris T. Suggs

Project Officer: Dr. Albert Kapikian

Objectives: Prepare research reference reagents against two strains of coronaviruses (OC 43 and 229E).

Major Findings: The master seed of OC 43 has been prepared and the master seed for 229E is presently in production.

Significance to NIAID Program and Biomedical Research: Since 16% of the lower respiratory tract infections are known to or caused by these two coronavirus strains reference reagents prepared against OC 43 and 229E would be very useful to the scientific community.

Proposed Course of Project: To be terminated June 1972

Date Contract Initiated: July 1971

Current Annual Level: $42,240

CENTER LABORATORIES (NIH-70-2226)

Title: Extraction of Antigen E from Dwarf Ragweed Ambrosia Elatior

Project Director: Mr. J. George Center

Project Officers: Dr. Charles Kirkpatrick and Mr. Robert M. Pennington

Objectives: To extract, process and package purified antigen E suitable for in vivo and in vitro immunological studies.

Major Findings: The bulk antigen E reagent had to be reprocessed after the initial purification. The purity was considerably below that stipulated in the specifications. A second purification was completed and shown to meet the criteria for potency and specificity. The purified antigen E was packaged for distribution and found to contain phenol. This product will be distributed for in vitro laboratory use only because of the contaminating phenol.

Significance to NIAID Program and Bio-Medical Research: Antigen E is chemically defined so that one could modify it and retest its antigenicity. There are at least thirteen ragweed antigens and only three have been characterized; antigen E, antigen K and antigen Ra 3. A highly purified and characterized homogeneous allergen may be used as a standard preparation for ragweed antigen E.

Proposed Course of Project: To be terminated October 1972

Date Contract Initiated: June 1970

Current Annual Level: Prior year support

WORTHINGTON BIOCHEMICAL CORPORATION (NIH-72-2067)

Title: Processing and Packaging of Antigen Ra 3

Project Director: Dr. Robert Flora

Project Officer: Mr. Robert M. Pennington

Objectives: To extract, purify and package ragweed allergen Ra 3 suitable for in vitro immunological studies.

Major Findings: The Ra 3 bulk allergen is presently being tested by the certification test laboratory.

Significance to NIAID Program and Biomedical Research: There are at least thirteen ragweed antigens and only three have been characterized; antigen E, antigen K and antigen Ra 3. A highly purified and characterized homogeneous Ra 3 allergen will be used as a standard reference preparation.

Proposed Course of Project: To be terminated April 1972.

Date Contract Initiated: January 1972

Current Annual Level: $7,500

ABBOTT LABORATORIES (NIH-71-2114)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Mr. George S. Marquis

Project Officer: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to 20 prototype rhinoviruses.

Major Findings: Three antigens have been approved for packaging and thirteen others are in production. The production of seven antisera has been initiated.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated in June 1972

Date Contract Initiated: April 1971

Current Annual Level: Prior year support.

CALIFORNIA STATE DEPARTMENT OF PUBLIC HEALTH (NIH-71-2112)

Title: The production and testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Schieble

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: Antigen production has been initiated on the five viruses assigned and immune serum production has been initiated on three.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated May 1972

Date Contract Initiated: March 1971

Current Annual Level: Prior year support.

SOUTHWEST FOUNDATION FOR RESEARCH AND EDUCATION (NIH-71-2110)

Title: The production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. S. S. Kalter

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to six prototype rhinoviruses.

Major Findings: Seed virus reagent production has been initiated on three and immune serum production has been initiated on two of the five viruses assigned.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated July 1972

Date Contract Initiated: April 1971

Current Annual Level: $5,135 (3 month extension)

UNIVERSITY OF VIRGINIA (NIH-71-2113)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Jack Gwaltney

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to five prototype rhinoviruses.

Major Findings: The production of two antigens has been initiated and two antisera are currently in production.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated July 1972

Date Contract Initiated: April 1971

Current Annual Level: Prior year support.

UNIVERSITY OF WISCONSIN (NIH-71-2111)

Title: The Production and Testing of Rhinovirus Research Reference Reagents

Project Director: Dr. Elliot Dick

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To prepare purified seed and corresponding antisera in guinea pigs to ten prototype rhinoviruses.

Major Findings: Production has been initiated on the ten antigens and three antisera are currently in production.

Significance to NIAID Program and Bio-Medical Research: These reagents are essential in bio-medical research due to their significant role in diseases of man. This virus group has been associated, along with the adenovirus and myxovirus groups with cold like respiratory diseases.

Proposed Course of Project: To be terminated October 1972

Date Contract Initiated: April 1971

Current Annual Level: Prior year support.

UNIVERSITY OF VERMONT (PH43-67-1188)

Title: To Test, Identify and Certify Rhinovirus Reference Reagents

Project Director: Dr. Charles A. Phillips

Project Officers: Dr. Albert Z. Kapikian and Mr. Robert M. Pennington

Objectives: To authenticate fifty-six Phase I rhinovirus reagents produced by Abbott Laboratories for identity, potency and purity. To authenticate Phase II rhinovirus reagents and any Phase I remakes to be produced by five production contractors.

Major Findings: To date forty-nine final vial Phase I antigens and 38 final vial Phase I antisera have been tested and approved for distribution. Seven seed viruses and two bulk antisera have been rejected because of low titer or contamination and are presently being remade.

The remaining sixteen Phase I final vial antisera are currently under test. Four bulk Phase II rhinovirus seeds have been approved for packaging.

Significance to NIAID Program and Bio-Medical Research: The contract furnishes authentication and purity confirmation on rhinovirus reagents being or to be produced by other independent contractors. The comprehensive certification testing will enhance the value of the rhinovirus reagents as reference standards.

Proposed Course of Project: The five contractors for the production of thirty-four Phase II rhinoviruses and any Phase I remakes are currently in production and as long as a contract is in effect for the production of rhinovirus reference reagents, the services of this contractor will be needed.

Date Contract Initiated: June 1967

Current Annual Level: $57,472

CENTER FOR DISEASE CONTROL

Title: Production of guinea pig antiserum to the Australia Antigen

Project Director: Dr. Morris T. Suggs

Project Officer: Dr. Robert C. Purcell

Objectives: To supply one liter of antisera to Au (a-y) subtype with a potency and specificity suitable for packaging and distribution as a reference reagent. The antisera will be prepared in guinea pigs.

Major Findings: The antisera has been prepared and is currently being tested by the certification laboratory prior to being packaged.

Significance to NIAID Program and Biomedical Research: Further research is needed to resolve the existence and character of hepatitis B subtypes. The availability of highly characterized sub-type antisera are valuable tools to the scientific community.

Proposed Course of Project: Production of Australia antigen research reference reagents to other sub-types (a - r) will continue. It is anticipated that larger quantities of diagnostic or survey reagents will be produced commercially or made available from other sources.

Date Contract Initiated: July 1971

Current Annual Level: $15,600

SMITHSONIAN INSTITUTION (PH43-67-59)

Title: Mammal Identification Service

Project Director: Dr. Charles O. Handley, Jr.

Project Officer: Mr. Robert M. Pennington

Objectives: To supply a mammal identification service center for NIH Intramural scientists, qualified government contractors and grantees.

Major Findings: From August 1971 through February 15, 1972, 2581 identifications were made.

Significance to NIAID Program and Bio-Medical Research: This contract has supplied a valuable service to the MARU in their epidemiological studies of hemorrhagic fevers.

Proposed Course of Project: The project will be reviewed in depth prior to renewal, with recommendations to be made to the Research Resources Committee concerning future activities or possible termination.

Date Contract Initiated: December 1, 1971

Current Annual Level: $38,785

Zonal Centrifuge Program

AEC - NIAID

Title: Molecular Anatomy (MAN) Program (Rockville and ORNL)

Project Director: Dr. Norman Anderson (Director, ORNL)

Project Investigator: Dr. John Gerin (Resident Director, Rockville)

Project Officers: Dr. Robert J. Byrne, Dr. George Galasso and Mr. Robert M. Pennington

Objectives: MAN (ORNL) - To develop biophysical and engineering methodology applicable to NIAID Programs. MAN (Rockville) - 1) to conduct collaborative research with NIAID Intramural scientists and collaborative research contractors; 2) To apply separation systems developed through MAN programs at Oak Ridge; 3) To train NIAID intramural scientists and collaborative research contractors in separation techniques.

Methods Employed: The MAN activity (ORNL) is engaged in the development of rotor systems and other biophysical instrumentation applicable to the NIAID programs. The Rockville facility evaluates centrifuge and biophysical instrumentation systems developed at ORNL and Rockville and applies them to specific projects of interest in the extramural and/or intramural programs of NIAID.

Major Findings: The various morphological forms of Au (22nm spherical and filamentous forms) each contain the group specific (a) and subtype specific (d or y, w or r) antigens and in approximately the same proportion.

Acrylamide gel electrophoresis (AGE) of the forms of a y antigen under reducing conditions reveals the same number of polypeptides in approximately the same proportion.

AGE analysis of purified a d antigen reveals 2 major and four minor proteins; the relative proportion of each protein is constant for each a d preparation analyzed.

AGE analysis of one a y antigen reveals an extra minor polypeptide.

Specific reagents to purified a d and a y antigens were prepared in guinea pigs; the anti- ad serum was adsorbed by affinity chromatography to remove trace quantities of anti-human sera albumin and is now being distributed by the Research Resource Branch; the anti-ay serum is currently being evaluated but all anti-human serum activity was removed by affinity chromatographic procedures in pilot experiments.

Studies to detect RNA dependent DNA polymerase activity in HB Ag purified by isopycnic banding and rate zonal procedures have been negative.

Structural studies of HB Ag suggest that HB Ag possesses hemagglutinin activity which results from the exposure of an antigen, possibly an

internal antigen, during the dissociation of the spherical particle.

Collaborative work with Saul Krugman has resulted in a purified ay antigen from a unit of blood from a Willowbrook patient; such a preparation will be considered for eventual use in active immunication to Type B hepatitis depending upon appropriate testing for safety and potency in susceptible animals.

Examination of HB Ag positive serum by immune electron microscopy (IEM) has revealed a significant proportion with evidence of circulating immune complexes, usually in antigen excess; IEM has revealed some evidence for an antigenic determinant (s) common to the Dane particle and filamentous forms not shared by the 22 nm spherical form.

Certain units of serum have been selected by IEM as particularly rich in the concentration of Dane particles and isolation of the Dane particles is presently in progress; preliminary studies are highly encouraging.

Serum rich in Dane particles with no evidence of antibody have been used in animal studies to establish an ay pool of known infectivity.

Biophysical and biochemical studies in collaboration with Dr. David Carver on the factor responsible for induction of hemadsorption negative areas in WI-38 cells have essentially dissociated the factor from HB Ag coat antigen, however critical studies are still in progress.

Other Studies Currently in Progress: Five inactivated influenza recombinant vaccines were prepared which are scheduled for or presently in clinical trials in order to assess the role of anti-neuraminidase antibody is protection to disease.

Basic studies with Respiratory Syneytial (RS) review has continued (a) a cell agglutination test (CAT) has been developed; (b) RS grown in HeLa cells has been labelled with H^3 uridine and C^{14} amino acid and partially purified by zonal centrifugation, (c) the virion associated with PML was purified from a diseased human brain for basic studies.

Significance to NIAID Program and Bio-Medical Research: The development and utilization of these separation systems provides a unique opportunity for the purification of viruses and virus subunits which will have application in the development of more effective vaccines. The program also affords the opportunity for training of NIAID and Collaborative contract scientists.

Proposed Course of Project: Due to the nature of the project and the rental agreement of the satellite facility it is estimated that the project will continue for at least four years based on the availability of funds.

Date Contract Initiated: April 24, 1964

Current Annual Level: $338,000

4. Publications

Field, J. and Kalter, S.S. Volume Production of Reference Seed Virus and Immune Ascitic Fluids for Six Arboviruses.

Gerin, J.L., Holland, P. V., Purcell, R.H. Australia Antigen; Large-Scale Purification from Human Serum and Biochemical Studies of its proteins. J. Virology, 1971, 7, 569-576.

Graham, B.J., Minamishima, Y., Dreesman, G.R., Haines, H.G., and Benyesh-Melnick, M. Complement-Requiring Neutralizing Antibodies in Hyperimmune sera to Human Cytomegaloviruses. J. Immunology, 1971, 107.

Haines, H.G., Von Essen, R., and Benyesh-Melnick, M., Preparation of specific antisera to cytomegaloviruses in goats. Proc. Soc. Exptl. Biol. & Med., 1971, 138, 846-849.

Schmidt, N.J., Melnick, J.L., Wenner, H.A., Ho, H.H., and Burkhardt, M.A. Evaluation of enterovirus immune horse serum pools for identification of virus field strains. 1971, Bull. Wld. Hlth. Org. 45, 317-330.

National Institute of Allergy and Infectious Diseases
Annual Report of the Assistant Scientific Director
for Laboratory and Clinical Research

The ten laboratories of the intramural program, including the Rocky Mountain Laboratory in Montana, Middle America Research Unit in the Panama Canal Zone, and the Pacific Research Section in Honolulu are concerned principally with the study of viral, bacterial and parasitic diseases. Considerable emphasis is also centered on immunologically mediated diseases. The organization and administration of these laboratories has remained essentially the same during fiscal year 1972. Overall direction of Laboratory and Clinical Research continued to be the broad responsibility of the Scientific Director, Dr. John R. Seal, while primary responsibility for administration of the various laboratories remained with the Assistant Scientific Director for Laboratory and Clinical Research, Dr. John E. Tobie.

The May 27-28, 1971, meeting of the Board of Scientific Counselors had not occurred by Annual Report time last year. This, as herein reported, was a review of the program of the Laboratory of Infectious Diseases which completed the deliberations of the Counselors on the three laboratories concerned with virological research. They considered that the Laboratory of Infectious Diseases was an outstanding example wherein the NIH has become a recognized center of excellence. Dr. Chanock, with his colleagues, is a leading contributor to research on the control and prevention of infectious diseases and the effectiveness of this Laboratory is due to the imaginative and capable leadership provided by its Chief. The Counselors stated that the emphasis on the natural history of acute infections of the respiratory and alimentary tracts and of hepatitis, directed toward the development of methods for the control or prevention of these infections, furnishes NIAID with the intramural competence necessary for it to serve as a national resource on these human health problems. The utilization of temperature sensitive mutants (ts) of RS virus, orthomyxoviruses, paramyxoviruses and mycoplasma for vaccine development seemed promising and the Board was impressed by the well ordered series of investigations to evaluate the utility of selected ts mutants for vaccine stocks. They praised the imaginative approaches for expediting detection and isolation of ts mutants. These constituted excellent examples of the ingenuity of the members of LID. Of particular interest were the recombination experiments in which ts mutants to A2 influenza were recombined with wild type Hong Kong virus. The recombinants of this cross had the ts characteristics of the A2 ts mutants and the hemagglutinin of the Hong Kong virus. Once an appropriate ts mutant with characteristics suitable for use as a live vaccine was developed and characterized, this mutant might be quickly recombined with a new influenza strain and a live attenuated vaccine strain with the antigenicity of the new strain thereby derived that would be in time to prevent a pandemic.

The Counselors considered the program on hepatitis to be especially meritorious in spite of the relatively small number of professional and technical level employees available to conduct their studies. The application of the specific radio-immunoprecipitation assay (RIP) for sensitive detection of the anti-Australia antigen (anti-HAA) was considered crucial for retrospective and prospective epidemiological investigations.

In an overview of the virus programs within the Laboratory of Biology of Viruses, Laboratory of Viral Diseases and Laboratory of Infectious Diseases, the Board felt that the research was truly outstanding. They were pleased with the extensive interactions, exchange of ideas and cooperation among these three laboratories, particularly by the younger professional staff. As training locations for medical research personnel, these laboratories are proven resources. The Board of Scientific Counselors felt that, in general, the virus research programs of NIAID were well-founded, vigorous, creative and had great relevance for human health.

Midway through December the Scientific Counselors met to review the immunologic research in NIAID's Bethesda laboratories, principally the Laboratory of Immunology (LI), Laboratory of Microbial Immunity (LMI), and Laboratory of Clinical Investigation (LCI). Dr. William Paul, with his colleagues in LI was considered to be an outstanding contributor to research on the regulation of the immune response. The Board was particularly impressed by the imaginative and capable efforts to correlate and interrelate the results of immunochemical, immunogenetic and immunobiological investigations. They were pleased to learn of the extensive collaborative studies between members within LI and between these staff members with immunologists in other laboratories and institutes. The Counselors especially recognized Dr. Paul as a stimulating leader who had created a strong esprit de corps and observed that the transition in leadership in LI had proceeded without interruption in the research and training programs of that Laboratory.

It was noted that Dr. Richard Asofsky, as Assistant Chief of the Laboratory of Microbial Immunity, had developed a productive program with a unique emphasis on the basic mechanisms in the induction of cellular and humoral immune responses to microbial and tissue antigens and in the pathogenesis of destructive lesions in homograft and autoimmune tissue reactions. The availability of a germfree facility with its animals in a protective environment was deemed central to much of the research of the Laboratory of Microbial Immunity.

Dr. Sheldon Wolff has developed a strong immunologic program in the Laboratory of Clinical Investigation that interrelates basic research with clinical investigation. The Board commented on the gratifying expansion of the immunologic program in LCI and the exciting nature of its present research. They recognized the importance of the Associate Program as being essential in the operation of LCI both for providing patient care and effecting productive research.

The extensive collaboration that has gone on between staff members of the Laboratory of Microbial Immunity with those in the Laboratory of Immunology has been most gratifying. The geographic proximity of the Laboratory of Immunology and the Laboratory of Clinical Investigation has facilitated the development of many cooperative efforts between these two laboratories and has led to significant advances in the Institute's immunological program. The Counselors viewed the conjoint seminars involving LI, LMI and LCI and between immunologists in NIAID and other institutes as most valuable.

For some time the intramural program has directed much effort toward the establishment of a program in clinical allergy. The results of these efforts will begin to be realized when Dr. Allen P. Kaplan, who is presently with Dr. Frank Austen, joins the Laboratory of Clinical Investigation in July 1972, to establish a Clinical Allergy Center within Laboratory and Clinical Research Because of the present personnel ceilings, it will be necessary to initiate this program on a somewhat modest scale. It is imperative that the Laboratory of Clinical Investigation acquire additional laboratory space on the 11th floor of the Clinical Center to properly house an expanding clinical allergy program. The Program must be of high quality and through the efforts of Dr. Kaplan must assume a position of leadership in development, research and training. Over the next decade it is foreseen that there must be considerable expansion so that it will become a truly outstanding Center for the study of allergic diseases.

The reduced personnel ceiling of NIAID has been the most serious problem of Laboratory and Clinical Research during the fiscal year. It has resulted in the inability of Laboratory Chiefs to effectively replace senior investigators as they were lost through attrition. The problem was further compounded in late October when the Institute was notified that its assigned ceiling for June 30, 1972, was 34 positions below that of last June. Unfortunately it was necessary for Laboratory and Clinical Research to absorb the entire personnel cut. Obviously further personnel reductions could not be made in most of the laboratories without doing irreparable damage to many ongoing productive programs. The Board of Scientific Counselors reviewed its previous assessments of the programs of the Middle America Research Unit and affirmed the outstanding quality of its program. However, in light of current personnel limitations and problems in maintaining high quality professional staff, the necessity of the Institute's decision to close the MARU installation was recognized. It has become more and more evident that personnel reductions must be achieved by selective action rather than by across-the-board measures. In the case of the Middle America Research Unit, its staff was at a minimum critical level and even a small reduction in the staff would have made the operation ineffectual. After the latter part of June 1972, MARU will no longer be an installation of Laboratory and Clinical Research, NIAID. However, the significant research as therein conducted will be logically phased out during the next fiscal year. In an effort to continue the research, MARU will be operated by the Gorgas Memorial Laboratory (GML) through a contract between NIAID and the Gorgas Memorial Laboratory until such time as GML obtains sufficient funds from Congress to cover the entire cost of such an operation. Certain of the present professional staff will be retained by NIAID during fiscal year 1973, and the majority of the technical personnel supported under the contract. This will permit a logical termination of the present studies and in the years to come will lead to an expanded and much strengthened program in tropical medicine in Panama and other Latin American countries as conducted by the Gorgas Memorial Laboratory.

Through the Associate Program, NIAID's Laboratory and Clinical Research continues to receive the appropriate stimulus provided by the flow of young physicians entering the various laboratories for periods of basic and clinical research. Their creative imagination and vigorous actions have proven

invaluable to many of the Institute's ongoing programs in immunological, virological and infectious disease research. The Institute and the NIH must take the necessary steps to assure the continued attraction of this significant group of scientists. Likewise the Staff Fellows have provided a challenge and stimulation to the senior professional staff and constitute an effective means for conducting high quality and very productive research. The majority of the Associates return to academic medicine since there is a demand throughout the country for young scientists trained by investigators of the caliber of NIAID's senior staff.

Although certain programs have been hampered by the personnel practices that have been imposed, the intramural scientists are to be congratulated on their overall ability to conduct up-to-date, vigorous, creative research of outstanding quality which is reflected in the following reports from the various laboratories of Laboratory and Clinical Research.

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Summary of Program
Laboratory of Clinical Investigation
July 1, 1971 - June 30, 1972

Sheldon M. Wolff, M. D., Chief of Laboratory
and Clinical Director, NIAID

Introduction

The Laboratory of Clinical Investigation has gone through a mild transitional period during the past year due to the departures of Drs. Johnson, Sheagren, Root and Song, as noted in last year's summary. With only the addition of Dr. Herbert Reynolds during the past year, we have been somewhat short-handed. However, this should be somewhat remedied during the coming year with the return of Dr. David Dale, who is presently Chief Resident, Department of Medicine, University of Washington Hospital, Seattle, Washington and Dr. Anthony Fauci, who is presently Chief Resident, Department of Medicine, the New York Hospital-Cornell Medical Center, New York, New York. Dr. Dale will continue his studies on the mechanisms that control granulocytes and the role of white blood cells in defense against infection. Dr. Fauci will reinstitute his studies on the pathogenesis of certain granulomatous diseases and the effect of immunosuppressive agents on immunological and inflammatory diseases.

I am very pleased to report that we have successfully recruited Dr. Allen Kaplan, presently at the Robert Breck Brigham Hospital, who will head a section on allergic diseases. Dr. Kaplan has done some outstanding work on the role of kinins in the inflammatory response and brings with him an excellent record of achievement in investigative medicine. He has received his training in allergy at the Robert Breck Brigham Hospital and undoubtedly will develop a strong program in clinical allergy. With his addition and the present staff in clinical immunology, our initial goals, when the present Clinical Director was appointed, for developing strong allergy and immunology programs will have been reached.

Unfortunately, I must report again this year the anticipated loss of two of our outstanding senior investigators. Dr. Harry Kimball, who for the past four years has ably run the clinical service in the Clinical Director's absence, will be leaving us at the end of June. Dr. Julius Kasel has accepted an appointment as Associate Professor of Microbiology, Baylor College of Medicine and will be leaving also this year. Dr. Kasel is the senior scientist in LCI in terms of service and both his and Dr. Kimball's departures will leave large voids in our programs. We are currently actively recruiting for a senior virologist to replace Dr. Kasel.

This past year has been one of increased productivity and recognition on an international and national scale for the members of LCI. For example, Drs. Frank, Kirkpatrick, Kimball and Wolff were invited to present papers at a WHO-sponsored meeting in Berne, Switzerland entitled, "Conference: A Re-Examination of 'Non-Specific' Factors Influencing Host Resistance." Dr. Rosenthal is a member of the organization committee of the Leukocyte Culture Conference and also attended a course at the Weizman Institute in Rehovoth, Israel entitled, "Biophysical Aspects of Antibodies and Cell Receptors." In addition, Dr. Rosenthal was invited to and presented a paper at the Concanavalin A meeting in Uppsala, Sweden. Dr. Bennett serves on the Advisory

Committee on Anti-Infective Agents for the Bureau of Drugs, Food and Drug Administration. Dr. Wolff was the Lilly Visiting Professor in South Africa and also was a Visiting Professor for the New Jersey College of Medicine, Cornell University, Good Samaritan Hospital in Phoenix, Arizona, amongst others. In addition, he is serving as Co-Chairman of an international conference on endotoxin to be held in June, 1972. Space does not permit us to list a number of other significant invitations received by members of LCI.

The continuing personnel restrictions, stringent fiscal requirements and space limitations have, despite the obvious productivity and growth of the laboratory, impeded the development of even greater strength of LCI. There is still considerable need for increasing the senior scientific staff and also to enlarge the number of support personnel presently employed. Space limitations will shortly become acute, and it is hoped that some relief will be forthcoming during the coming fiscal year.

Research Accomplishments

The Laboratory continues to place major emphasis on studies relating to clinical immunology, virology, mycology and host defense mechanisms. The role and function of blood cells as major determinants of resistance to infections are extensively investigated.

LYSOSOMES

Density gradient separation of human neutrophil granules has documented the presence of three distinct classes of granules which differ markedly in their enzyme content and morphology. Eosinophils have a single granule class rich in peroxidase and several other hydrolases but lacking lysozyme and alkaline phosphatase. Granules from patients with CHS have different sedimentation properties as well as quantitative abnormalities in their enzymes and, unlike normals, CHS leukocytes fail to release lysosomal enzymes during phagocytosis. (West, Kimball).

CHEMOTAXIS

Defects in cell migration have been documented in leukocytes from patients with the Chediak-Higashi syndrome, paroxysmal nocturnal hemoglobinuria, and systemic lupus erythematosus. The majority of SLE sera will not generate normal amounts of chemotactic factors, a finding that appears to be unrelated to complement levels. Urea inhibits the formation of serum chemotactic factors, a finding which accounts for the deficit observed in uremic sera. Serum chemotactic activity has been separated by gel filtration into two distinct cleavage fragments of C5, when generated by either the classical or recently described alternate complement pathways. During phagocytosis pulmonary alveolar macrophages have been shown to release factors capable of generating active chemotactic factors from either serum or bronchial secretions. A highly sensitive radiolabeling technique for studying chemotaxis has been developed which will yield more information about the kinetics, efficiency and quantification of directed leukocyte migration.(Clark, Kimball).

CELLULAR IMMUNITY

The mechanisms of host defense against infection with chronic intracellular parasites are under study using an *in vitro* model of cellular immunity. This system has allowed investigation of the immunological reactions leading to activation of bactericidal mechanisms in the guinea pig peritoneal exudate macrophage. Such activation depends on the presence of antigenically stimulated, sensitized lymphocytes. The nature of the lymphocyte-macrophage interaction is now under study.(Simon).

WHITE CELL FUNCTION

As compared to the neutrophil, the eosinophil has been shown to be hypermetabolic with respect to glucose metabolism, H_2O_2 production, and peroxidase activity. It does not kill bacteria as well because it is not as effective in phagocytosis. Further, unlike the neutrophil, peroxidase plays no role in the bactericidal mechanism(s) of the eosinophil. Neutrophils of some patients with paroxysmal nocturnal hemoglobinuria do not phagocytize bacteria normally and thereby do not kill an inoculum of *Staphylococcus aureus* as well as normal neutrophils.

Guinea pig peritoneal macrophages which have been immunologically activated acquire an enhanced bactericidal capability against facultative intracellular organisms by a mechanism that does not involve biochemical pathways classically associated with the production of H_2O_2.(Bujak).

NEUTROPENIA

Studies have been done in patients with cyclic neutropenia and they appear to have cyclical fluctuations in most of the formed elements of the blood, not just neutrophils. Thus, the human disease is similar to that in the grey collie dog, which has been previously shown by members of LCI to have cyclical hematopoiesis. Patients with malaria who are neutropenic have been shown to have decreased marrow reserves and very prolonged granulocyte survival times. The neutropenia is due to a markedly enlarged marginal granulocyte pool with a diminished circulating pool.(Guerry; Dale, Dep't. of Med., Univ. of Washington Hospital, Seattle, Wash.; Wolff).

A defect of cell maturation has been detected at the stage of second granule formation in the developing marrow neutrophil. The appearance of such a lesion corresponds to the cyclic fluctuation of marrow differentiation in the grey collie. This defect, cytoplasmic autophage, glycogen aggregate deposits, accumulation of membrane vesicles suggest an autoregulatory defect of genetic origin in these dogs.(Dale; Rosenthal; Wolff; Scott, Univ. Minn., Dep't. Path.).

NONSPECIFIC RESISTENCE

The administration of iron to mice can completely reverse the protection afforded the mice to *C. albicans* and pneumococcal infections by endotoxin. Endotoxin administration to human beings results in their sera inhibiting growth of *C. albicans in vitro*.(Elin, Wolff).

GRANULOMATOUS DISEASES

Therapy of Wegener's granulomatosis patients with immunosuppressive agents continues to be excellent. These patients have been shown to have increased levels of secretory IgA, which may have pathogenic importance. Recent studies have shown, for the first time, that some of these patients have antigen-antibody deposits in their kidneys.(Fauci, Dep't. of Med., the New York Hosp.-Cornell Medical Center, New York, N. Y.; Rosenthal, Wolff).

HYDROCORTISONE AND CELL-MEDIATED IMMUNITY

Hydrocortisone has been assumed to alter clinical disease states through lympholysis. It has been observed that hydrocortisone blocks an in vitro correlate of cell-mediated immunity (CMI), the assay of macrophage inhibitory factor (MIF). This blockade is not the result of failure of antigen uptake on the processing macrophage, recognition by immune thymus derived lymphocyte or synthesis of the lymphokine, MIF. Rather, the data indicate that steroids block the effect of MIF on the target macrophage either by interference with a surface membrane receptor or by inducing an intracellular metabolic event antagonistic to the decreased migration of macrophages. These data identify for the first time a specific in vitro site of hydrocortisone action in CMI. The studies provide insight into the therapeutic modes by which steroids alter in vivo host defenses to external challenge. In addition, these studies suggest a mechanism by which this drug may alter spontaneous or viral induced autochthonous CMI reactions against self.(Balow, Rosenthal).

LYMPHOCYTES

The lymphocytes within the peritoneal exudate (PELS) appear to represent thymus-derived or T-lymphocytes. At least two types exist. One possesses a cell surface membrane modification, a uropod, whose formation is metabolically dependent. B lymphocytes which bear surface immunoglobulin do not appear to form uropods. Activation of immunocompetent T-lymphocytes is a complex phenomenon. While the exact mechanism is not established, the data strongly indicate a multi-step event in which an initial interaction of antigen with an immunologically naive macrophage occurs,followed by T-cell macrophage interaction and, hence, antigen recognition. T-lymphocytes do not themselves appear capable of direct interaction with soluble antigen. Regression analysis of cell density and studies employing metabolic inhibitors indicate a necessary physical interaction between the immunospecific T-lymphocyte and the macrophage being immunorelevant antigen.(Rosenstreich, Rosenthal).

Immunoglobulin and, thus, presumably antigen specific receptors exist tightly bound to the surface membrane of B-lymphocytes. T-lymphocytes by contrast lack easily detectable surface immunoglobulin. On interaction with antigen or antibody directed against these determinants, migration of these receptors towards the centrosome associated cell pole occurs followed by internalization. While binding to these receptors occurs at 0-4°C, migration on contact with a multivalent ligand requires expenditure of cell energy for "capping" and internalization. However, the clinging or aggregation of these prescribed receptors occurs within the plane of the cell membrane even in the presence of metabolic inhibitors. This phenomenon has been studied at the ultrastructural level using a

rabbit anti-guinea pig immunoglobulin reagent coupled to horseradish peroxidase (RAGIG-HRPO). Studies of the fate of internalized receptor with time is being pursued.(Rosenthal, Davie, Paul).

The interaction and fate of concanavalin A, a plant lymphocyte phytomitogen, with the target cell, has been investigated. EM studies as well as biochemical studies show a complex activation event both in terms of kinetics and differential responsiveness to Con A and PHA, indicating heterogeneity of T-lymphocytes. Using Con-A-Cytochrome-C peroxidase complexes, Con A was localized to patches of the cell surface.(Rosenthal, Davie, Paul).

CELLULAR IMMUNITY IN CRYPTOCOCCOSIS

Studies continued on cellular immunity in cryptococcosis. Macrophages derived from human blood monocytes were found to ingest and kill encapsulated cells of Cryptococcus neoformans even in the absence of complement and opsonins. Macrophages from cryptococcosis patients had no apparent defect in intracellular killing.(Diamond, Bennett).

FIVE-FLUOROCYTOSINE

Five-fluorocytosine (5-FC) is a new, commercially available oral antifungal drug that is either used alone or in conjunction with amphotericin B. By developing a bioassay that distinguished accurately between the two antifungal drugs, higher blood levels of 5-FC were found in patients receiving combined therapy compared to patients receiving 5-FC alone. Further studies in patients and rabbits clearly showed that the elevation was due to amphotericin B-induced azotemia. The assay proved helpful in choosing 5-FC dosages that would be adequate but would not encounter excessive toxicity as a result of combined therapy. (Block, Bennett).

SCHISTOSOMIASIS

The largest single category of patients admitted to hospital were cases of schistosomiasis, with the remainder comprising a wide variety of known and suspected parasitic diseases. The schistosomiasis patients are generally lightly infected as judged by quantitative egg counts of urine or feces and only one or two have shown abnormalities of organ structure or function. A circulating antigen that can be detected by gel diffusion or immunoelectrophoresis in plasma of heavily infected mice or hamsters could not be detected in serum of 27 heavily infected Brazilian cases. Yet, two of five NIH patients exhibited antibody to this circulating antigen. The malaria studies, conducted primarily on infected volunteers in Atlanta but also on a few NIH cases, have demonstrated temporary depression of serum complement during paroxysms of a relapse (eight cases) but not during early paroxysms of the primary attack (twelve cases). (Franck, Nash, Neva).

ENTAMOEBA HYSTOLYTICA

Recognition of viruses of Entamoeba hystolytica latent in amebic cell lines maintained axenically for long periods of time in the laboratory prompts an investigation of their role, if any, in the pathogenesis of human amebiasis.

Two new isolates from patients with amebic disease are currently being examined for the presence of virus, and additional isolates from symptomless carriers as well as patients with clinical amebiasis will be studied. The biology of the virus-ameba relationship, methods of purifying virus from cell components, virus classification by serology and host range, and cloning of ameba in an attempt to produce virus-free cell lines are under investigation. (Franck).

COMPLEMENT

Studies in C4 deficient guinea pigs have defined the role of two different pathways of complement activation. The classical pathway functions most efficiently in cytotoxic reactions; the alternate pathway is more efficient in inflammatory responses and protection from infection. It has been possible to show that antibodies to tissue components will not produce immunologic injury by activation of the alternate pathway. Studies of Forssman shock have shown that the alternate pathway cannot produce death following injection of anti-Forssman antibody (an antibody to a guinea pig tissue antigen). If the components of the classical pathway are present, death is produced by a reaction which takes place in the tissues rather than in the intravascular space as formerly believed. (Frank, May, Kane).

HEREDITARY ANGIONEUROTIC EDEMA

A double-blind study of the therapeutic effectiveness of epsilon amino caproic acid has shown that this drug is highly useful in the treatment of hereditary angioneurotic edema. Some new aspects of possible clinical toxicity have been defined. Studies of the level of C4 in blood and spinal fluid in patients with suspected CNS lupus have demonstrated that CNS C4 may provide important information about the pathophysiology of this disease. (Frank, Gerwin, Kane).

AUTOIMMUNE HEMOLYTIC ANEMIA

Studies of the role of antibody and complement in the immune clearance and destruction of erythrocytes have shown that IgM and IgG immunoglobulins activate different mechanisms of clearance. In the case of IgM, antibody-coated erythrocytes are sequestered by the liver, held in the liver for a period of time and then released back into the circulation as Coombs-positive cells. IgG causes progressive splenic sequestration of the cells with no release into the circulation once the cells are trapped. The effect of splenectomy and steroid therapy are presently under investigation. (Frank, Atkinson, Kane).

MUCOCUTANEOUS CANDIDIASIS

Patients with chronic mucocutaneous candidiasis have provided substantial evidence for some mechanisms of defective cellular immunity. Preliminary work has provided some guidelines for restoration of immune competence. Current efforts are concentrated on evaluation of "transfer factor," because this material appears to be free from adverse effects. (Kirkpatrick).

INFLUENZA

In human adult subjects, the presence of serum anti-neuraminidase antibody was found to be associated with a significant reduction of illness and virus

excretion following experimental challenge with influenza virus. The degree of host resistance was correlated with the level of antibody at the time of challenge. Such findings indicate that induction of anti-neuraminidase antibody followed by natural or experimental exposure with influenza virus could result in a more durable and lasting immunity. Initial data from studies in man with an inactivated vaccine containing a recombinant virus having an irrelevant hemagglutinin and relevant neuraminidase stimulated relatively high titers of antineuraminidase antibody. (Kasel).

ADENOVIRUS

The finding that crystallized preparations of adenovirus type 5 hexon and fiber antigens stimulate neutralizing antibodies without adverse vaccine reactions overcomes the objections associated with the use of whole virus vaccines. The possibility now exists to provide highly effective vaccines against adenoviral disease for infants and children. (Kasel).

SECRETORY IMMUNOGLOBULINS

Efforts have been directed toward isolation of large quantities of canine secretory piece and intestinal IgA which will permit the detailed structural analysis, amino acid composition and sequence. The susceptibility of dimeric serum and secretory IgA to proteolytic enzymes is being studied. Additionally, the interactions of gut bacterial antigens and processing cells (T-lymphocytes) in gut associated lymphoid tissue (Peyer's patches) are being investigated. (Reynolds, Levin).

PSEUDOMONAS INFECTIONS

The effect of antibodies, both circulating and secretory, on the bactericidal and phagocytic activity of pulmonary macrophages was studied in rabbits immunized with Pseudomonas aeruginosa. The Pseudomonas vaccine was given for one month; then the animals were bled and their respiratory macrophages and bronchial secretions obtained. Mean titers of agglutinating antibody in immune sera were 1:256 and in bronchial secretions 1:16. The serum titers were unchanged for three months while the bronchial secretion titers decreased after two months. Fractionation of sera and sucrose density gradient ultracentrifugation of sera and secretions showed serum antibody activity to be predominantly IgG and solely IgG in secretions. Short-term cultures of pulmonary macrophages were established and phagocytic and bactericidal activities of these cells were measured by constructing killing curves after preopsonized ^{14}C-labeled Pseudomonas had been added to the macrophage monolayers. When immune bronchial secretions or IgG were used to opsonize Pseudomonas, ^{14}C uptake by macrophages was increased by > 50%; the number of macrophages that phagocytized organisms was increased by 15 to 20%, especially those which ingested multiple organisms. Immune IgA and IgM were inferior opsonins as compared with IgG. Thus, immune bronchial secretions through the effects of IgG opsonic antibody enhanced phagocytosis and the bactericidal capabilities of respiratory macrophages. (Reynolds, Pennington).

Serial No. NIAID-10(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Office of the Chief

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4-5/12			4-5/12
Professional:	3/12			3/12
Other:	4-2/12			4-2/12

Project Description:

Objectives:

To develop, direct and coordinate the program of the Laboratory, as defined in the individual research projects; where necessary, to redirect individual projects to meet current needs and advances in the field; to align clinical projects with patient availability; to provide an unexcelled standard of patient care for patients utilized in research.

Methods Employed:

Organize available staff and recruit qualified personnel, both professional and sub-professional, to develop the program and carry out its aims; maintain close liaison with area medical societies, institutions and individuals for referral of patients whose diagnoses fall within the active or proposed disease research categories; the highest standards of patient care are maintained by selection of qualified physicians; the development of necessary policies and continued close and direct supervision of patient care activities; professional consultant services to other institutes in the area of infectious diseases; continued guidance of research projects undertaken by younger investigators; maintenance of staff morale.

Proposed Course:

To continue

Publications:

1. Fauci, A. S. and Johnson, J. S.: Antigenic competition at the cellular level: response of rabbit lymph node cells to the trinitrophenyl and p-Arsanilic haptens. J. Immun. 106: 1396-1398, 1971.

2. Fauci, A. S. and Johnson, J. S.: Suppression of antibody synthesis. I. Evidence for a circulating inhibitor of antibody synthesis demonstrable at the cellular level. J. Immun. 107: 1052-1056, 1971.

3. Fauci, A. S. and Johnson, J. S.: Suppression of antibody synthesis. II. The effect of carrier-specific cells upon haptenic competition. J. Immun. 107: 1057-1062, 1971.

4. Reynolds, H. Y. and Johnson, J. S.: Structural units of normal canine serum and secretory immunoglobulin A. Biochemistry 10: 2821-2827, 1971.

5. Grant, J. A., Harrington, J. T. and Johnson, J. S.: Carboxy-terminal amino acid sequences of canine immunoglobulin G subclasses. J. Immun. 108: 165-168, 1972.

Serial No. NIAID-11(c)
1. LCI
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Clinical Care Program in Support of Research Projects

Previous Serial Number: SAME

Principal Investigators: Dr. Sheldon M. Wolff
Dr. DuPont Guerry, IV

Other Investigators: Dr. John P. Atkinson
Dr. Charles A. Dinarello
Dr. John I. Gallin
Dr. Fred A. Gill
Dr. Douglas M. Levin
Dr. Peter E. Lipsky
Dr. James E. Pennington
Dr. Arthur R. Rabson
Dr. Michael S. Toren

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5-7/12		1-3/12	6-10/12
Professional:	4-6/12		1-3/12	5- 9/12
Other:	1-1/12		0	1- 1/12

Project Description:

Responsibility for care of patients in the various research areas.

Proposed Course:

To continue.

Publications: NONE

Serial No. NIAID-12(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanisms of Fever and Host Responses

Previous Serial Number: SAME

Principal Investigator: Dr. Sheldon M. Wolff

Other Investigators:
Dr. Harry R. Kimball
Dr. Ronald J. Elin
Dr. Harvey B. Simon
Dr. Joseph S. Bujak
Dr. DuPont Guerry, IV
Dr. Alan S. Rosenthal
Dr. Charles A. Dinarello
Dr. John I. Gallin
Dr. Michael M. Frank
Dr. Burton C. West
Mr. Stanley B. Ward
Mr. Joseph B. Edelin

Cooperating Units:
Dr. David W. Alling, OSD, NIAID, NIH (no project no.)
Dr. Paul D. Berk, MET, NCI, NIH (no project no.)
Dr. Terrence F. Blaschke, MET, NCI, NIH (no project no.)
Dr. David C. Dale, University of Washington Hospital, Department of Medicine, Seattle, Washington
Dr. Anthony S. Fauci, the New York Hospital-Cornell Medical Center, Department of Medicine, New York, New York
Dr. Harvey R. Gralnick, CP, CC, NIH (no project no.)
Dr. Robert I. Henkin, ETB, NHLI, NIH (no project no.)
Dr. Robert Horn, Vanderbilt Univ. School of Medicine, Department of Pathology, Nashville, Tennessee
Dr. Ralph E. Johnson, GL&C, NCI, NIH (no project no.)
Dr. Seymour Perry, OASDP, C, NCI, NIH (no project no.)
Dr. Robert E. Scott, Univ. of Minnesota School of Medicine, Department of Pathology, Minneapolis, Minnesota
Dr. Elliot Vesell, M. S. Hershey Medical Center, Department of Pharmacology, Hershey, Pennsylvania

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	7-1/12			7-1/12
Professional:	6			6
Other:	1-1/12			1-1/12

Project Description:

Selected patients are being studied on the wards and in the laboratory. The patients can be divided into the following groups: patients with a) recurrent fever; b) Chediak-Higashi syndrome; c) granulomatous disorders; d) malaria; e) cyclic and other neutropenias; f) eosinophilia; and g) infectious mononucleosis; and normal volunteers.

Extensive studies are in progress relating to the pathogenesis of fever, granulocyte function, immune and other responses of humans and experimental animals to infections, antigens, etc.

Objectives:

Human Studies:

1. To assess varied biologic responses of normal and a variety of patients to etiocholanolone.

2. The effects of pyrogens on the release of vasoactive peptides (kinins) and lysosomal enzymes are under investigation.

3. To assess the responses of humans to multiple doses of endotoxin (endotoxin tolerance).

4. To intensively study groups of patients with such poorly defined illnesses as familial Mediterranean fever, recurrent fever, Chediak-Higashi syndrome, midline granuloma, Wegener's granulomatosis, cyclic and other neutropenias in an attempt to better delineate the clinical syndrome.

5. To study the mechanisms responsible for the release of endogenous pyrogen.

6. To characterize and purify leukocytic pyrogen.

7. To assess the effect of immunosuppressive agents, in particular cyclophosphamide, on human diseases.

8. To study the mechanisms responsible for control of leukocyte levels in health and disease.

9. To define the primary and secondary features of the Chediak-Higashi syndrome.

10. To explore the pathogenesis of the increased susceptibility to infection in a variety of diseases.

11. To study what humoral factors, if any, are responsible for the granulocytopenias seen in certain diseases of man.

12. To determine the marrow granulocyte reserves in neutropenic states and to perfect better tests for this parameter.

13. To investigate leukokinetics in patients with depressed and elevated granulocyte counts.

14. To characterize the colony stimulating activity found in the urine of neutropenic patients.

15. To study the effect of fever on drug metabolism.

16. To determine the effect of endotoxin and etiocholanolone on coagulation and iron metabolism in man.

Animal Studies:

1. To define the features of the beige mouse which are similar to the Chediak-Higashi syndrome of man.

2. To determine the susceptibility of beige mice to infection.

3. To assess the protective effects of endotoxin in mice challenged with various mycotic organisms and, if possible, to characterize the humoral and cellular factor(s) responsible for protection.

4. To further characterize the pyrogenic and hematologic reactivity of immunized rabbits challenged with specific antigen.

5. To examine the role(s) of endogenous and leukocytic pyrogen and soluble antigen-antibody complexes in the production of experimental immune fever.

6. To investigate the reaction between endotoxin and lysates from the horseshoe crab.

7. To characterize the humoral factors responsible for endotoxin tolerance.

8. To study what humoral factors, if any, are responsible for the cyclic granulocytopenia seen in grey collie dogs.

9. To better define the grey collie syndrome.

10. To determine the marrow granulocyte reserves in grey collie dogs.

11. To determine the kinetics of circulating granulocytes in dogs with depressed and elevated granulocyte counts.

12. To study the role of colony stimulating activity found in dog urine.

13. To investigate the growth of C. albicans in human sera.

Methods Employed:

Patients and volunteers receive varying doses of endotoxin and etiocholanolone. Rectal temperatures are taken and a variety of responses are determined: antibody levels, immunoglobulins, plasma cortisol, growth hormone, serum iron, clotting factors, etc.

The mobilization of peripheral granulocytes in response to etiocholanolone, endotoxin, or cortisol is studied in patients before, during and after chemotherapy.

Basic studies of immune mechanisms in dysproteinemias, Chediak-Higashi syndrome, etc., include quantitative immunoglobulin determinations using agar diffusion methods, immunoelectrophoresis, paper electrophoresis, complement levels, levels of antibody by the bentonite flocculation technique, and ability of individuals to develop delayed hypersensitivity through challenge with dinitrochlorobenzene on the skin.

Mice are given endotoxin and subsequently challenged at varying time intervals with such infectious agents as Candida albicans, Plasmodium berghei, etc. Donor mice are exsanguinated; the whole sera or fractions are passively transferred to normal mice, which are then challenged with the infectious agent. Iron is given to mice to study its protective role against infections.

Normal New Zealand NIH rabbits are immunized with nonpyrogenic human serum albumin (HSA) or keyhole limpet hemocyanin using a series of intravenous inoculations. Following an appropriate rest period, antibody levels are measured in the serum using bentonite flocculation and precipitation reactions. Soluble antigen-antibody complexes are formed following precipitation of antibody at equivalence by the addition of sufficient antigen in either normal or immune serum.

An automatic temperature recorder is used to obtain rectal temperatures at regular intervals before and after challenge with antigen, complexes or pyrogen. Hematological responses are evaluated by analysis of ear vein blood samples obtained at various times preceeding and following challenge. Circulating pyrogen is assayed by passively transferring serum from challenged, immunized animals into normal recipients. Buffy coat leukocytes are separated from whole, heparinized rabbit blood following centrifugation and washed in phosphate buffered saline. Counts are made on aliquots of the washed leukocytes, and various concentrations of the agents under study are added to the cell suspensions. After incubation for a specific time interval, leukocytic pyrogen is measured by injection of supernatants into normal and endotoxin-tolerant recipient rabbits, and febrile responses are obtained.

To determine what humoral factors are present in neutropenic states, bone marrow cultures are done in both autologous and isologous plasma. Leukokinetic studies are done using radiophosphate-labeled white blood cells.

Bone marrow cells are cultured in vitro, and the activity of human and dog urine to stimulate colony formation is measured.

Patient Material:

Patients are admitted to the Clinical Center, as are normal volunteers, for these studies. In addition, patients from other Institutes participate in these studies.

Major Findings:

Therapy of the accelerated phase of the Chediak-Higashi syndrome with vincristine and prednisone continues to be encouraging.

Patients with recurrent fever of unknown etiology have been found to represent a diverse array of clinical disorders. For example, 13 such patients have been found to have granulomatous hepatitis, while ten others have what we are calling adult Still's disease.

Patients with malaria and infectious mononucleosis have (temporarily) neutropenia, diminished marrow granulocyte reserves and markedly prolonged granulocyte survival times in the peripheral blood.

Iron can reverse some of the protective effects of endotoxin when given to mice infected with C. albicans or Pneumococci.

Cyclic neutropenia may be a more general cyclical defect in hematopoiesis and, therefore, be like the dog model.

Endotoxin causes a significant increase in factor 8 in human beings.

The inflammatory response (Rebuck skin window technique) is depressed only when significant granulocytopenia is present.

Resistance to infection in neutropenic patients may be due to the numbers of monocytes present.

Colony stimulating activity (possibly leukopoietin) cycles in grey collie dogs and patients with cyclic neutropenia.

Prednisone by mouth or intravenous cortisol in addition to etiocholanolone and endotoxin are potent stimuli for the release of granulocytes from the marrow granulocyte reserves.

BSP clearance is significantly prolonged in experimental fever in man.

Grey collie dogs have cyclic neutropenia which recurs every 12 days. During the neutropenic phase, marrow granulocyte reserves are depressed. Serum muramidase level fluctuates cyclically and reaches its lowest point about 24 hours before the peripheral granulocyte count is at its lowest level. The presence of myeloid precursors in the bone marrow also follows a cyclical pattern as does the incorporation of tritiated thymidine into bone marrow cells.

Other cells (lymphocytes, reticulocytes, platelets) besides granulocytes also cycle in grey collie dogs, suggesting that the defect in these animals is due to a regularly recurring defect in stem cells.

Cyclophosphamide has produced striking remissions and clinical improvement in patients with Wegener's granulomatosis.

Radiation therapy has proven to be remarkably effective therapy for midline granuloma.

The growth of C. albicans in human sera is inversely proportional to the pH and directly related to the iron saturation in human sera.

Beige mice are more susceptible to C. albicans infection than control mice.

Significance to Biomedical Research and the Program of the Institute:

The large number and wide variety of studies listed above provide considerable new knowledge dealing with host response mechanisms to a wide variety of clinical and experimental diseases and the pathogenesis of fever.

Proposed Course:

The above outlined studies are to be continued and some areas are to be expanded.

Honors and Awards:

Dr. Wolff received the Superior Service Award, NIH. He was the Eli Lilly Visiting Professor in South Africa for 1971. He was a Visiting Professor at the New Jersey College of Medicine; Cornell University Medical Center; Department of Medicine, Good Samaritan Hospital, Phoenix, Arizona; Memorial Medical Center, Savannah, Georgia; Department of Microbiology, University of Arizona, College of Medicine, Tucson, Arizona; and Infectious Diseases Unit, University of Rochester School of Medicine and Dentistry, Rochester, New York.

Publications:

1. Perry, S. and Wolff, S. M.: Neutropenia. In Conn. H. F. (ed.): Current Therapy. Philadelphia, W. B. Saunders Co., 1971, pp. 223-224.

2. Root, R. K., Wolff, S. M., and Baer, H.: Skin reactivity of human subjects to a polysaccharide component of BCG culture filtrates. Proc. Soc. Exp. Biol. Med. 136: 403-406, 1971.

3. Dale, D. C., Brown, C. H., Carbone, P., and Wolff, S. M.: Cyclic urinary leukopoietic activity in grey collie dogs. Science 173: 152-153, 1971.

4. Dale, D. C. and Wolff, S. M.: Skin window studies of the acute inflammatory responses of neutropenic patients. Blood 38: 138-142, 1971.

5. Mickenberg, I. D., Snyderman, R., Root, R. K., Mergenhagen, S. E., and Wolff, S. M.: Immune fever in the rabbit: responses of the hematologic and complement systems. J. Immun. 107: 1457-1465, 1971.

6. Mickenberg, I. D., Snyderman, R., Root, R. K., Mergenhagen, S. E. and Wolff, S. M.: The relationship of complement consumption to immune fever. J. Immun. 107: 1466-1476, 1971.

7. Fauci, A. S., Wolff, S. M., and Johnson, J. S.: Effect of cyclophosphamide upon the immune response in Wegener's granulomatosis. New Eng. J. Med. 285: 1493-1496, 1971.

8. Mickenberg, I., Root, R., and Wolff, S.: Bactericidal and metabolic properties of human eosinophils. Blood 39: 67-80, 1972.

9. Wright, L. J., Lipsett, M. B., Ross, G. T., and Wolff, S. M.: Effects of dexamethasone and aspirin on the responses to endotoxin in man. J. Clin. Endocr. 34: 13-17, 1972.

10. Dale, D. C., Alling, D. W., and Wolff, S. M.: Cloxacillin chemoprophylaxis in the Chediak-Higashi syndrome. J. Infect. Dis. 125: 393-397, 1972.

11. Wolff, S. M., Dale, D. C., Clark, R. A., Root, R. K., and Kimball, H. R.: The Chediak-Higashi syndrome: studies of host defenses. Ann. Intern. Med. 76: 293-306, 1972.

12. Steinberg, A. D., Plotz, P. H., Wolff, S. M., Wong, V. G., Agus, S. G., and Decker, J. L.: Cytotoxic drugs in treatment of nonmalignant diseases. Ann. Intern. Med. 76: 619-642, 1972.

13. Blume, R. S. and Wolff, S. M.: The Chediak-Higashi syndrome: studies in four patients and a review of the literature. Medicine In press.

14. Gralnick, H. R., Blume, R. S., and Wolff, S. M.: Leukocyte protease deficiency in Chediak-Higashi syndrome. Blood In press.

15. Dale, D. C. and Wolff, S. M.: Cyclic neutropenia in man and grey collie dogs. Birth Defects In press.

16. Dale, D. C., Alling, D. W., and Wolff, S. M.: Cyclic hematopoiesis: the mechanism of cyclic neutropenia in grey collie dogs. J. Clin. Invest. In press.

17. Herzberg, J. H. and Wolff, S. M.: Chronic factitious fever in puberty and adolescence: a diagnostic challenge to the family physician. Psychiatry in Medicine In press.

Serial No. NIAID-14(c)
1. LCI
2. Medical Virology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Medical Virology - Clinical Investigations in Viral Infections and Diseases

Previous Serial Number: SAME

Principal Investigator: Dr. Julius A. Kasel

Other Investigators: Dr. Michael S. Toren
Miss Margret Huber

Cooperating Units: Dr. Robert Chanock, LID, NIAID (Project No. 66(h))
Dr. Robert B. Couch, Baylor College of Medicine, Houston, Texas

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2-9/12			2-9/12
Professional:	2-5/12			2-5/12
Other:	4/12			4/12

Project Description:

Objectives:

1. To define and evaluate immunologic factors which contribute to host resistance to viral infections.

2. To investigate the feasibility of employing purified structural viral proteins and recombinant viruses that have been developed in the laboratory as vaccines for the control of disease in man.

3. To define the etiologic agent as non-bacterial gastroenteritis.

Methods Employed:

Human adult volunteers are immunized or challenged with a variety of experimentally produced vaccines or infectious inocula. Clinical responses are closely followed by attending physicians. Laboratory studies include an assessment of humoral and cellular immune responses by appropriate immunologic procedures.

Dissolved crystalline preparations and protein structural subunits of adenovirus are obtained by biophysical and biochemical procedures.

Antigenic hybrids are obtained by in vitro recombination experiments with human and animal influenza viruses.

Major Findings:

Influenza Virus:

Results from studies in man strongly suggest that serum anti-neuraminidase antibody, in addition to antihemagglutinin antibody plays an important role in host resistance to influenza virus. Experimental virus challenge of adult volunteers lacking serum antihemagglutinin antibody but possessing antineuraminidase antibody was associated with a significant reduction in illness response and virus excretion. Moreover, the degree of immunity was found to be correlated with the level of antibody titer at the time of virus challenge. In other studies, an experimental influenza virus vaccine (X-32) prepared with an antigenic hybrid containing an irrelevant hemagglutinin (H-equi-1) and neuraminidase of human origin ($N2_{HK}$) was shown to be immunogenic in man. The levels of serum antineuraminidase antibody were comparable to those observed under natural conditions of exposure to influenza virus.

An analysis of the relative frequencies of antibodies to the envelope proteins of H-equi-2 N-equi-2 in persons of various ages revealed that most of the antibody reactions to both antigens occurred in persons born before 1900. This data and results from studies of others indicate that sometime in the past man experienced infection with an influenza virus immunologically similar to that isolated from horses in 1963.

Adenovirus:

Crystallized preparations of adenovirus type 5 structural protein subunits were found to be immunogenic and relatively non-toxic in adult subjects. Parenteral administration of aqueous vaccines was followed by the induction of two types of serum neutralizing antibody.

Nonbacterial Gastroenteritis:

Studies in adult volunteers indicate that acute nonbacterial gastroenteritis is probably caused by a small virus sharing properties with those of the parvovirus group.

Significance to Biomedical Research and the Program of the Institute:

The recognition that antineuraminidase antibody modifies the host response to influenza virus suggests an approach to immunization for the control of influenza. The induction of such antibody by vaccines containing recombinant viruses followed by natural or experimental exposure with attenuated virus could result in a more durable and longer lasting immunity.

Acceptable adenovirus vaccines for pediatric use are not currently available. Stimulation of neutralizing antibody without adverse reactions

in adults by dissolved crystalline adenoviral subunits overcomes the objections associated with the use of whole virus vaccines. It now may be feasible to provide highly effective vaccines against adenoviral disease for children.

Proposed Course:

It is planned to continue and expand the program as stated under objectives.

Honors and Awards: NONE

Publications:

1. Fedson, D.S., Fulk, R.B., Huber, M.A., Reisberg, M.A., and Kasel, J.A.: Antineuraminidase antibody response in serum and nasal secretions following intranasal or subcutaneous inactivated A2/HongKong/68 influenza virus vaccine. J. Immun. 107: 730-737, 1971.

2. Couch, R.B., Douglas, R.D. Jr., Fedson, D.S., and Kasel, J.A.: III. Protection against experimental influenza in man. J. Infec. Dis. 124: 473-480, 1971.

3. Kasel, J.A., Couch, R.B., and Douglas, R.D. Jr.: Antigenicity of alum and aqueous adenovirus hexon antigen vaccines in man. J. Immun. 107: 916-919, 1971.

4. Rossen, R.D., Kasel, J.A., and Couch, R.B.: The secretory immune system: Its relation to respiratory viral infection. Med. Prog. Virol. 13: 194-238, 1971.

5. Fedson, D.S., Huber, M.A., Kasel, J.A., and Webster, R.G.: Presence of A/Equi-2 hemagglutinin and neuraminidase antibodies in man. Proc. Exp. Biol. Med. 139: 825-826, 1972.

6. Dolin, R., Blacklow, N.R., DuPont, H., Buscho, R.F., Wyatt, R.G., Kasel, J.A. Hornick, R., and Chanock, R.M.: Biological properties of Norwalk agent of acute infectious non-bacterial gastroenteritis. Proc. Exp. Biol. Med. In press.

7. Murphy, B.R., Kasel, J.A., and Chanock, R.M.: Association of serum anti-neuraminidase antibody with resistance to influenza in man. New Eng. J. Med. In press.

8. Rossen, R.D., Sell, K.W., Kasel, J.A., Shulman, N.R., Jora, J.J., Judd, K.P., Trentin, J.J., and Bulter, W.T.: Preparation of antilymphocyte globulin for human use: Attempts to find a combination of antigen cell dose and adjuvant which reliably provides immunosuppressive horse anti-lymphocyte globulin. Transplantation. In Press.

Serial No. NIAID-18(c)
1. LCI
2. Clinical Mycology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Systemic Fungal Disease

Previous Serial Number: SAME

Principal Investigator: Dr. John E. Bennett

Other Investigators: Dr. Richard Diamond
Dr. Edward Block

Cooperating Units: Miss Anne Jennings, CP, NIH (Project Number 63)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5-5/12			5-5/12
Professional:	2-11/12			2-11/12
Other:	2-6/12			2-6/12

Project Description:

Objectives:

1. Study host defense mechanism in human cryptococcosis.

2. Improve diagnosis and therapy of human systemic mycoses.

Methods Employed:

Purified blood monocytes and neutrophils were separated from peripheral blood by centrifugation in a Hypaque-Ficoll gradient. Monocytes were converted to macrophages by cell culture. Phagocytosis of C. neoformans was measured by microscopic examination and uptake of ^{32}P-labeled cryptococci. Intracellular killing was measured by plate counts. Leukocytes were separated for lymphocyte transformation by sedimentation of heparinized human peripheral blood. Lymphocyte transformation was measured by uptake of tritiated thymidine in cultures.

A bioassay was devised for measuring amphotericin B and 5-fluorocytosine separately in the body fluids of patients taking both drugs. Biologic activity of 5-fluorocytosine was ablated by addition of cytosine to the amphotericin B assay medium. Ultrafiltration of the sample removed amphotericin B activity completely but had no effect on 5-fluorocytosine, which then could be assayed in the ultrafiltrate.

Sensitivity of fungi to 5-fluorocytosine was assayed by a tube dilution technique using cytosine-free broth.

Major Findings:

1. Cellular immunity in cryptococcosis:

Studies were completed on phagocytosis and killing by human peripheral blood neutrophils and monocytes. The major findings were in last year's report. In addition, it was found that hydrogen peroxide alone could kill C. neoformans in vitro. Neutrophils can attain intracellular peroxide concentrations equal to or greater than those used in these studies. Hydrogen peroxide generation may then provide an additional cryptococcal killing mechanism in vivo.

Studies proceded on killing by human macrophages. After 3 or more days in culture, macrophages avidly ingested cryptococci in the presence of heat inactivated human serum. In contrast, freshly separated monocytes required heat-labile factors (presumably complement components) for C. neoformans ingestion. Macrophages cultured for varying time intervals were used to measure cryptococcal killing. There were no significant differences in ingestion or killing of cryptococci comparing 17 cryptococcosis patients and 12 normal controls. Attempts were made to specifically activate macrophages by culturing them with autologous lymphocytes and specific soluble antigens, including cryptococcin, streptokinase-streptodornase (SK-SD), and tuberculin PPD. Such antigen treated cells morphologically resembled "activated macrophages." As expected from their increased surface area, these cells ingested more organisms. However, increased killing of C. neoformans or Listeria monocytogenes by such cells could not be demonstrated.

Available soluble cryptococcal antigens proved to be weak stimuli for in vitro lymphocyte transformation. However, 10^5-10^8 heat-killed cryptococci added to cultures consistently stimulated high levels of uptake of tritiated thymidine. In 4 normal laboratory workers previously exposed to cryptococci, 10^7 C. neoformans produced a mean tritiated thymidine uptake of 86,737 dpm (range 40,922-169, 564 dpm). Uptake by their unstimulated control cells was only 1230 dpm (range 841-1670 dpm). Thus far, we have also studied 5 successfully treated cryptococcosis patients who had no known predisposing illnesses and were not receiving corticosteroid or immunosuppressive therapy. In two, there was little or no stimulation of thymidine uptake by cryptococci (183 and 1,680 dpm) or SK-SD. Three others had a normal response to SK-SD, but peak responses to cryptococci were lower than those of the above controls (13741, 22575, and 37584 dpm). This preliminary data suggests that at least some of the cryptococcosis patients without obvious predisposing factors may have defects in lymphocyte function. Further studies are needed to confirm and clarify the nature of such defects.

2. Studies with 5-fluorocytosine (5-FC):

Bioassay. Serum and cerebrospinal fluid concentrations of 5-FC were

measured in 15 patients in order to define the dose response. Special techniques (see Methods) allowed documentation that amphotericin B-induced azotemia profoundly elevated 5-FC serum levels. In the rabbit, decreased renal but not hepatic function altered 5-FC catabolism.

Drug resistance. A study of patients with cryptococcosis revealed 1 out of 36 pretreatment and 10 of 15 post-treatment isolates to be resistant to 5-FC. Among the 22 patients treated here, 5 out of the 10 treatment failures could be accounted for by the development of drug resistance. Drug resistant colonies were induced easily in vitro and shown by fluctuation analysis to be not drug induced but to be spontaneously appearing mutants. Such mutants were found in 16 of 18 pretreatment isolates at a frequency of 0.01 to 17 per 10^6 wild type cells. In experimental murine cryptococcosis, a slight but statistically significant synergism between amphotericin B and 5-fluorocytosine was measured. This synergism was shown to be due, at least in part, to a decreased incidence of 5-fluorocytosine resistance arising during therapy.

Mechanism of action. Clinically obtainable concentrations of 5-FC prevented replication but did not decrease viability of C. neoformans. Nor did the durg depress utilization of ^{14}C-glucose or oxygen by sensitive strains. Preliminary studies with ^{14}C-leucine have suggested impaired protein synthesis. Drug resistance appeared as a single step mutant with extremely high tolerance to the drug. Such mutants were also resistant to 5-fluorouracil, suggesting that resistance was not due to repression of cytosine deaminase.

Studies on candidiasis and cladosporiosis. Among 11 pretreatment isolates of Candida albicans, all were sensitive to 5-FC. Post-treatment isolates were not studied. Cerebral cladosporiosis is usually fatal in man. Studies in experimental murine cladosporiosis have shown 5-FC to be the first effective chemotherapeutic agent ever described for this infection.

3. Serologic tests for the diagnosis of cryptococcosis:

The section has continued to test serum and cerebrospinal fluid specimens sent from outside hospitals. In the previous 12 months 506 such specimens were run. This effort is done because it is a public service, a means of obtaining newly diagnosed patients with cryptococcosis, and a means of expanding our experience with the clinical value of these tests.

Signficance to Biomedical Research and the Program of the Institute:

1. A great deal of attention within the Institute as well as elsewhere has been focused on macrophage-mediated immunity. The majority of the work is being done in animals. By studying the role of the human macrophage in cryptococcosis, we not only hope to elucidate more fully the mechanisms of host response to that disease, but also to contribute to the understanding of human macrophage function in general.

2. The antifungal drug, 5-fluorocytosine, is now commercially available. Yet, many basic questions about the pharmacology of the drug remain unknown.

The studies described here should help provide a more sound basis for dealing with the problems of clinical usage.

Proposed Course:

Studies on cellular immunity in cryptococcosis will continue. Although it is clear that macrophages can kill cryptococci it is not clear whether macrophages activated by lymphokines are better able to kill cryptococci, or, for that matter, better able to kill any other microorganism. Future studies will be directed at discovering the optimum system for activating human macrophages with sensitized lymphocytes and cryptococcal antigens. Then, killing of cryptococci will be measured using activated macrophages from cryptococcosis patients and normal volunteers.

Studies on 5-fluorocytosine will be continued and expanded to investigations of tissue penetration and protein binding.

Honors and Awards:

Dr. Richard Diamond -
Member - Advisory Committee on Anti-Infective Agents, Food and Drug Administration.

Dr. John Bennett -
Member - Editorial Board, Antimicrobial Agents and Chemotherapy.
Consultant - to Anti-Infective Agents Division, Bureau of Drugs, Food and Drug Administration.
Lecturer - Department of Microbiology, Johns Hopkins University School of Medicine.

Publications:

1. Bennett, J. E.: Bay 5097, New drug for systemic mycoses? (Editorial) Ann. Intern. Med. 73: 653, 1970.

2. Young, R. C. and Bennett, J. E.: Invasive aspergillosis: absence of detectable antibody response. Amer. Rev. Resp. Dis. 104: 710-716, 1971.

3. Bennett, J. E. and Bailey, J. W.: Control for rheumatoid factor in the latex test for cryptococcosis. Amer. J. Clin. Path. 56: 360-365, 1971.

4. Diamond, R. D., Root, R. K. and Bennett, J. E.: Factors influencing killing of Cryptococcus neoformans by human leukocytes in vitro. J. Infect. Dis. 125: 367-376, 1972.

5. Bennett, J. E.: Cryptococcosis. In Hoeprich, P. (Ed.) Infectious Diseases. Harper and Row, Inc. In press.

6. Block, E. and Bennett, J. E.: Pharmacological studies with 5-fluorocytosine. Antimicrob. Agents Chemother. In press.

7. Young, R. C., Jennings, A. and Bennett, J. E.: Species identification of invasive aspergillosis in man. Amer. J. Clin. Path. In press.

8. Jennings, A. E. and Bennett, J. E.: The isolation of red yeast-like fungi in a diagnostic laboratory. J. Med. Micro. In press.

Serial No. NIAID-23(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 throught June 30, 1972

Project Title: Observations on the Canine Immunoglobulins

Previous Serial Number: SAME

Principal Investigator: Herbert Y. Reynolds

Other Investigators: Dr. Douglas Levin

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1 7/12			1 7/12
Professional:	11/12			11/12
Other:	8/12			8/12

Project Description:

Objectives:

1. Completion of the construction of a molecular model for canine secretory IgA by identifying the assembly and interactions of secretory piece and J-chain.

2. In addition the ultrastructure of secretory piece and in vitro study on the susceptibility to proteolysis of dimeric serum and secretory IgA are to be examined.

3. Examination of gut associated lymphoid tissue (Peyer's patches) in new born dogs for its response to bacterial antigens and subsequent capabilities of processing the antigens and of immunoglobulin (antibody) production.

Methods Employed:

Gel filtration and ion exchange chromatography; standard techniques of zone electrophoresis, immunodiffusion; sedimentation velocity and equilibrium ultracentrifugation; equilibrium dialysis and fluorescence quenching for determination of binding energies; liquid scintillation spectroscopy; polyacrylamide disc gel electrophoresis. Intestinal lymphoid cell tissue cultures autoradiography; immunofluorescence and ^{3}H thymidine stimulation of lymphocytes will be used.

Major Findings:

Secretory piece (SP), predominately attached to colostral and intestinal IgA through convalent (disulfide) bonding, has been dissociated, and isolated and its molecular weight of approximately 50,000 daltons established. Further identification of J-chain, using polyacrylamide separation techniques, is in process. Further characterization of SP with amino acid analysis and sequence awaits the purification of a larger amount of appropriate glycoprotein. Likewise, intestinal IgA (Reynolds and Johnson, J. Immuno. 4: 888, 1970), is being prepared in batch quantities for use in proteolysis experiments and for use in antibody purification with Sepharose immunoadsorbents.

Peyer's patch lymphocytes have been isolated and successfully maintained in tissue culture for periods exceeding 7 days which has been a formidable technical problem considering the bacterial contamination present on the gut surfaces. The lymphocytes, which have been identified as approximately 80% B-type and 20% T-types, have shown unexpected responsiveness to such stimulants as phytohemagglutin with more rapid and higher ^{3}H thymidine uptake than comparable cell populations from spleens and lymph nodes. These observations are still quite preliminary.

Significance to Biomedical Research and the Program of the Institute:

These studies defining the molecular models of canine IgA's have made critical testing possible for the role of secretory piece in protecting secretory IgA from proteolysis and in a transport function. J-chain, likewise, is important in the assembly of IgA and probably IgM molecules. All these studies of canine IgA help to extend the homology of the mucosal secretory defense system in mammals and humans which is recognized as a major barrier for interaction with viral and bacterial micro-organisms by the host.

Studies on the function of gut lymphoid cells in birds and mammals, such as the Peyer's patch areas, are contradictory and may point to the tremendous species variations which will prevent easy generalities about function and significance. Therefore, each mammalian species will ultimately require study. The interaction of lymphoid cells which process antigens, such as gut bacterial antigens, and those which subsequently produce appropriate antibodies are liekly to be vital in regulating the growth of normal intestinal flora and in the development of intestinal illnesses such as cholera and other diarrheal diseases.

Proposed Course:

1. Further work on the structure of secretory piece and J-chain to show the interactions with IgA immunoglobulin and the elucidation of the chains ultrastructure.

2. The function of antigen processing cells in gut lymphoid tissue using new born puppies with surgically created intestinal loops (Thiry—Vella loops) for in vivo experiments to extend in vitro cell culture observations.

Honors and Awards: NONE

Publications:

1. Reynolds, H. Y., and Johnson J.S.: Structural units of normal canine serum and secretory IgA. Biochemistry, 10: 2821, 1971.

Serial No. NIAID-27(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host Defense Mechanisms

Previous Serial Number: SAME

Principal Investigator: Dr. Harvey B. Simon

Other Investigators: NONE

Cooperating Units: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	5/12			5/12
Professional:	4/12			4/12
Other:	1/12			1/12

Project Description:

Objectives:

To study the mechanisms responsible for the production of enhanced resistance to infection with chronic intracellular parasites using an in vitro model of cellular immunity.

Methods Employed:

Strain II guinea pigs are immunized with bovine gamma globulin (BGG), picrylated human serum albumin (PIC-HSA) or tubercle bacilli in complete Freund's adjuvant. Cutaneous delayed hypersensitivity to the specific antigen is verified after 2-6 weeks. Sterile peritoneal exudates from immune and control animals are induced by injection of light mineral oil. After three days, the peritoneal exudates are harvested and placed in short term tissue culture in the presence or absence of antigen. After 24 hours, the macrophage monolayers are washed, and infected with standardized inocula of Listeria monocytogenes. At serial intervals, duplicate monolayers are washed and stained or lysed and quantitatively cultured by the pour plate technique.

Macrophage migration inhibition assays are performed on aliquots of these peritoneal exudates using standard techniques.

Purified lymphocytes are obtained from immune and control animals by incubating peritoneal exudate cells or lymph node cells on packed viscose rayon columns.

Major Findings:

1) Macrophages from guinea pigs immune to BGG, PIC-HSA, or tuberculin acquire a markedly enhanced ability to kill an immunologically unrelated microorganism, Listeria monocytogenes, when whole peritoneal exudates from these animals are cultured overnight in the presence of specific antigen.

2) The immunologic specificity of this reaction depends on the presence of sensitized lymphocytes. Antigenically stimulated immune lymphocytes can enhance the bactericidal capacity of normal macrophages in vitro.

3) Peritoneal exudate lymphocytes are more potent in this assay than lymph node lymphocytes.

4) Normal macrophages are not activated by immune serum.

5) Macrophage migration inhibition occurs in parallel with macrophage bactericidal enhancement when whole lymphocytes are tested. However, cell-free supernate preparations with a high degree of MIF activity have not enhanced macrophage bactericidal activity in this system, suggesting that another, as yet unrecognized, mediator may be involved in the bactericidal reaction.

Significance to Biomedical Research and the Program of the Institute:

Immunity to infections with facultative intracellular parasites (e.g. tuberculosis, brucella, deep mycotic infections and many parasitic and probably viral infections) seems to be mediated primarily through the macrophage. Our findings to date further relate the phenomenon of acquired cellular immunity to delayed hypersensitivity, and our in vitro system will enable its mechanism to be thoroughly elucidated.

Proposed Course:

The project will be discontinued.

Honors and Awards: NONE

Publications:

1. Simon, H. B. and Sheagren, J. N.: Cellular Immunity in vitro I. Immunologically mediated enhancement of macrophage bactericidal capacity. J. Exp. Med. 133: 1377-1389, 1971.

2. Simon, H. B. and Sheagren, J. N.: Enhancement of macrophage bactericidal capacity by antigenically stimulated immune lymphocytes. Cellular Immunology. In press.

Serial No. NIAID-28(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Inflammation

Previous Serial Number: SAME

Principal Investigator: Dr. Harry R. Kimball

Other Investigators: Dr. Robert A. Clark
Dr. Burton C. West
Dr. John I. Gallin
Dr. Ethel Patten
Dr. Sheldon M. Wolff
Dr. Alan S. Rosenthal
Dr. Michael M. Frank
Dr. Joseph S. Bujak
Dr. Herbert Y. Reynolds

Cooperating Units: Dr. John Decker, NIAMD (Project No. NIAMD-ARB-10c)
Dr. David C. Dale, University of Washington Hospital, Seattle, Washington
Dr. John Marshall, UCLA Medical Center, Los Angeles, California
Dr. Kenneth Melmon, University of California, San Francisco, California
Dr. George Padgett, Washington State University, Pullman, Washington

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4 2/12			4 11/12
Professional:	2 10/12		9/12	3 7/12
Other:	1 4/12			1 4/12

Project Description:

Objectives:

1) Study the activity and distribution of lysosomal acid hydrolases in normal granulocytes and in the leukocytes of patients with Chediak-Higashi syndrome (CHS) and other leukocyte disorders.

2) Study of lysosomal enzymes in lymphocytes, monocytes, eosinophils and activated macrophages in both man and animals and correlate with specific functions.

3) Separate and identify different classes of granulocytes granules (lysosomes) of both normal and abnormal leukocytes and evaluate their biochemical and morphologic characteristics.

4) Determination of the effects of steroids, estrogens, chloroquine, bacterial products, antigen-antibody complexes on intact granulocyte lysosomes and their surrounding membrane.

5) Determination of intracellular distribution and function of lysosomal contents of normal and CHS leukocytes during phagocytosis and lymphocyte transformation and correlate with morphologic characteristics.

6) Study of in vitro leukocyte migration (chemotaxis) of normal and CHS leukocytes as well as granulocytes from patients with recurrent infections and correlate with in vivo inflammatory responses.

7) Study leukocyte migration in altered metabolic states of uremia, acidosis, hepatic coma.

8) Study of origin, nature and adequacy of serum chemotactic factors in normals and patients with compromised host defenses.

9) Evaluate generation of serum chemotactic factors in hypocomplementemic animals (C4 deficient guinea pigs) and man (SLE).

10) Study in vitro and in vivo chemotaxis in CHS mink, cattle and beige mice (animal model of human CHS).

11) Identification and study of chemotactic factors in normal and abnormal human leukocyte lysosomal granules.

12) Develop new methods for the study of active and passive leukocyte migration, determine effects of various chemical agents on chemotaxis.

13) Study release of chemotactic factors in phogocytozing leukocytes and alveolar macrophages.

14) Study of serum and marrow levels of lysozyme and establish its role as an indicator of granulocyte turnover and production in normal and leukopenic man as an cyclic neutropenia of dogs.

15) Study the vasoactive peptides ("kinins") in the inflammatory response to certain pyrogens.

16) Investigate the effects of estrogens and androgen on etiocholanolone fever.

Methods Employed:

Peripheral blood leukocytes are separated into granulocyte and mononuclear fractions by gradient centrifugation in a Hypaque-Ficoll solution. Granulocytes are washed, lysed in heparin and sucrose to achieve full breakage of intact cells. Differential centrifugation is utilized to obtain granule rich fractions which are then studied for biochemical and morphologic (phase and EM) characteristics. Assays of the lysosomal enzymes, acid phosphatase (para nitrophenyl phosphate and beta glycerophosphate substrates), alkaline phosphatase, beta glucuronidase, lysozyme, myeloperoxidase, cathepsins are used as lysosomal markers. Assays of protein and non-lysosomal malic dehydrogenase are performed. Granule fractions are further separated by zonal or iospynic density gradient centrifugation into their various classes and studied for their biochemical and morphologic properties. Isolated leukocyte granules are incubated with various stabilizing agents, separated by high speed centrifugation, and release of enzymatic content measured in the supernatant fractions.

Rebuck skin windows are utilized to measure leukocyte migration in man. A modification of the Boyden chamber technique is used to study chemotaxis in vitro. Dextran sedimented leukocytes are prepared for the chemotactic chamber and chemotactic factors are generated by incubating human or guinea pig serum with bacterial filtrates, endotoxins of antigen-antibody complexes.

Patients and volunteers are given adrenal steroids, estrogens and graded doses of etiocholanolone. Precise temperature responses are recorded and blood is obtained for measurement of marrow granulocyte reserves and plasma kinin levels.

Major Findings:

1) Reproducible methods have been developed for the further separation of pure neutrophils and their granules from human blood.

2) Normal human neutrophil granules are clearly separable into three biochemically and morphologically distinct populations. These conform to primary, secondary and tertiary granules classes identified in animal leukocytes.

3) Giant CHS granules have lowered activities of myeloperoxidase (25% of normal), beta glucuronidase (45% of normal), PNP acid phosphatase (60% of normal) and muramidase (80% of normal). Cathepsin D and BGP acid phosphatase levels are normal while alkaline phosphatase is 2-fold higher. The intracellular distribution of these enzymes differs from normal.

4) Phagocytizing CHS leukocytes fail to release their lysosomal contents normally either to the extracellular media or to the intracellular phagocytic vacuole.

5) CHS leukocytes do not respond to chemotactic factors normally (reduced to approximately 70% of normal), and one patient with CHS was found to have a marked deficiency in generating serum chemotactic factors.

6) A defect in cellular chemotaxis documented in CHS mink (animal model of CHS) which closely parallels that seen in CHS humans. This offers an animal model for the study of normal and abnormal cell migration.

7) A cell defect in chemotaxis has been identified in a child with recurrent and unexplained infections.

8) Serum generation of chemotactic factors is reduced in uremia and certain patients with SLE (unrelated to azotemia). Cell function remains normal.

9) A new radiolabel technique has been devised for the study of leukocyte chemotaxis.

10) C4 deficient serum generates normal amounts of chemotactic activity by the alternate complement pathway although this reactive is less rapid than in normal serum utilizing the classical complement sequence.

11) Serum chemotactive activity generated by endotoxin or immune complexes resides in two distinct C5 cleavage products.

12) Phagocytizing alveolar macrophages release a factor capable of activating serum or bronchial secretions to produce neutrophil chemotactic factors.

13) Leukocytes from some patients with PNH demonstrate abnormal phogocytosis, killing and cell migration.

14) Free "kinin" levels are increased in the plasma of patients with known endotoxemia.

15) Serum muramidase parallels the production of marrow granulocytes both in man and dogs with cyclic neutropenia.

Significance to Biomedical Research and the Program of the Institute:

Leukocyte lysosomes and their enzymes play an important part in the pathogenesis of certain inflammatory diseases. Most of this work has been done in experimental animals and conclusions inferred to the human pathologic state. Our purpose is to study human lysosomes, primarily from leukocytes, and establish their importance in both normal and abnormal inflammatory processes. The CHS is of special interest since this is a disease in which lysosomal mechanisms do not function properly. Defective migration of these cells to an inflammatory site in concert with lower activities of certain bactericidal enzymes and a faulty intracellular delivery sistem has major consequences for host resistance in these patients.

Detailed knowledge of the biochemical nature of these granules and their contribution to the varied function of granulocyte will further clarify the nature of the CHS defect and increase our understanding of normal leukocyte function in the inflammatory process.

Proposed Course:

It is planned to continue some of the studies outlined above.

Honors and Awards: NONE

Publications:

1. Clark, R. A., and Kimball, H. R.: Defective granulocyte chemotaxis in the Chediak-Higashi syndrome. J. Clin. Invest. 50: 2645-2652, 1971.

2. Wolff, S. M., Dale, D. C., Clark, R. A., Root, R. K., and Kimball, H. R.: The Chediak-Higashi syndrome. Ann. Int. Med. 76: 293-306, 1972.

3. Kimball, H. R., Melmon, K. L., and Wolff, S. M.: Endotoxin induced kinin production in man. Proc. Soc. Exp. Biol. Med. 139: 1078-1082, 1972.

4. Wolff, S. M., Kimball, H. R., and Clark, R. A.: The Chediak-Higashi syndrome. In: Clinical Delineation of Birth Defects. In press.

5. Clark, R. A., Kimball, H. R., and Padgett, G. A.: Granulocyte chemotaxis in the Chediak-Higashi syndrome of mink. Blood. In press.

6. Dale, D., Ward, S. B., Kimball, H. R., and Wolff, S. M.: Studies of neutrophil production and turnover in grey collie dogs with cyclic neutropenia. J. Clin. Invest. In press.

Serial No. NIAID-30(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Interaction of Antibody and Complement in the Production of Immune Damage

Previous Serial Number: SAME

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Joseph May
Dr. Michael Kane
Miss Thelma Gaither
Dr. Robert Gerwin

Cooperating Units: Dr. Leonard Ellman, LI, NIAID (NIAID-142)
Dr. Ira Green, LI, NIAID (NIAID-142)
Dr. Richard Root, University of Pennsylvania, Philadelphia, Pennsylvania

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2 3/12			2 4/12
Professional:	2 2/12		1/12	2 3/12
Other:	1/12			1/12

Project Description:

Objectives:

1. To determine the role that each of the various immunoglobulin classes play in complement mediated cytotoxic reactions, complement fixation and generation of biologically active products from complement components.

2. To evaluate the role of complement in immunologic diseases.

Methods Employed:

Quantitative complement component assay, preparation of intermediates in the complement sequence, various complement components on the surface of cells and purification of complement components and antibodies to the various classes are routinely used in this laboratory. These methods employ the use of DEAE cellulose and C-M cellulose, Sephadex, electrophoretic techniques and ultracentrifugation for the purification of immunoglobulins and complement component proteins. Cellular intermediates in the complement sequence (i.e. cells

sensitized with antibody and reacted with various components of complement) are prepared by our own modification of standard methods and are used for titration of complement components. Efforts are made to determine the interrelationships and efficiency of various complement components reacting with other components from the same species and cross species lines. Purified preparations of various antibodies, antigens and endotoxins are used to study immunologic responsiveness of a new strain of guinea pigs recently established in our laboratory with a total deficiency of C4, one of the early reacting components in the complement sequence.

Major Findings:

The C4 deficient guinea pig strain, developed in cooperation with Drs. Ellman and Green has been used to study the pathogenesis of immune damage. C4 represents the second complement protein in a series of nine proteins capable of interacting with antigen antibody complexes and destroying cells and tissues. This new strain represents the first deficiency in any animal of a complement component which acts early in the complement sequence and is the only available genetic deficiency of any component in the guinea pig. These animals therefore present a major opportunity to discover the role of complement in the body's defenses and the function of complement in disease. Our major findings are as follows: Many of the inflammatory responses which are classically thought to be complement dependent are found to be intact in this new strain of guinea pigs. The animals have an intact Arthus reaction and intact passive cutaneous anaphylaxis reactions. They have a mild defect in their antibody response to several antigens, but this is not universally true for all antigens. They manifest normal delayed hypersensitivity to several antigens. The animals have a marked defect in the immune clearance of antibody coated, chromated red blood cells. A series of in vitro studies have therefore been performed to determine why these animals are capable of showing many complement mediated reactions in the total absence of C4. These studies indicate that the C4 deficient animals have a well developed by-pass mechanism to by-pass the early components of the complement sequence. Thus, they do not utilize C1, C4 and C2 to produce many inflammatory reactions. Use of their serum allows for the clear demonstration of this new pathway which has been postulated to exist in normal serum. These studies provide the first conclusive evidence for the existence of such a pathway and the first deomonstration its operation in vivo. These studies have also shown that the classical complement pathway (C1, C4 and C2) is required for the complement mediated immune damage of cells by antimembrane antibody both in vivo and in vitro.

Significance to Biomedical Research and the Program of the Institute:

These studies provide a foundation for understanding the biochemical events which lead to tissue damage in many autoimmune diseases. The information is essential to the rational development of chemotherapy for these illnesses.

Proposed Course:

The biological role of the alternate and classical complement pathways will be explored in greater detail. The interaction between complement, endotoxins, and clotting factors will also be evaluated.

Honors and Awards: NONE

Publications:

1. Ellman, L., Green, I., Judge, F. and Frank, M. M.: In vivo studies in C4 deficient guinea pigs. J. Exp. Med. 134: 162-175, 1971.

2. Frank, M. M., May, J. Gaither, T. and Ellman, L.: In vitro studies of complement function in sera of C4 deficient guinea pigs. J. Exp. Med. 134: 176-187, 1971.

3. Shevach, E., Herberman, R., Leiberman, R., Frank, M. M. and Green, I.: Receptors for immunoglobulin and complement on mouse leukemias and lymphomas. J. Immunol. 108: 325-334, 1972.

4. Frank, M. M., Ellman, L. and Green, I.: A new genetically-controlled complement abnormality: C4 deficiency in the guinea pig. Biological Activities of Complement, Basel, Karger, 1972, pp. 256-259.

5. Root, R. K., Ellman, L. and Frank, M. M.: Bactericidal and opsonic properties of C4 deficient guinea pig serum. J. Immunol. In press.

6. May, J. and Frank, M. M.: Complement mediated tissue damage-contribution of the classical and alternate complement pathways in the Forssman reaction. J. Immunol. In press.

7. Shevach, E., Herberman, R., Frank, M. M. and Green, I.: Receptors for complement and immunoglobulins on human leukemic cells and human lymphoblastoid cell lines. J. Clin. Invest. In press.

8. Sandberg, A. L., Synderman, R., Frank, M. M. and Osler, A. G.: Production of chemotactic activity by guinea pig immunoglobulins following activation of the C3 complement shunt pathway. J. Immunol. In press.

Serial No. NIAID-33(c)
1. LCI
2. Clinical Allergy and Hypersensitivity Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of Delayed Hypersensitivity

Previous Serial Number: SAME

Principal Investigator: Dr. Charles H. Kirkpatrick

Other Investigators: Mr. Terrill K. Smith

Cooperating Units: Dr. Nils Axelsen, Protein Laboratory, University of Copenhagen, Copenhagen, Denmark
Dr. J. C. Meek, Kansas University Medical Center, Kansas City, Kansas
Dr. Lauren Packman, Children Memorial Hospital, Chicago, Illinois

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1 11/12			1 11/12
Professional:	1 6/12			1 6/12
Other:	5/12			5/12

Project Description:

Objectives (Sub-project A)

1. Characterization of cellular immunity in patients with chronic mucocutaneous candidiasis.

Methods Employed:

Peripheral blood lymphocytes from healthy normal volunteers have been studied in short term culture with antigens from C. albicans and phytohemagglutinin (PHA). The kinetics of dose-response relationships have been determined by labelling cells with ^{3}H-thymidine and counting incorporated radioactivity.

Production of the lymphokine, macrophage migration inhibition factor (MIF), has been studied in cultured peripheral blood lymphocytes from patients with chronic mucocutaneous candidiasis. This assay involves exposure of lymphocytes to antigens in vitro, collection of culture fluids which are concentrated, and assayed for their capacity to inhibit migration of guinea pig macrophages.

The in vitro studies are correlated with in vivo cellular immunity as determined by skin testing with 7 antigens known to eilicit delayed cutaneous responses.

Major Findings:

Sixteen patients with chronic mucocutaneous candidiasis have been studied. Delayed skin responses have identified three immunological categories. Four patients had normal delayed reactions to C. albicans as well as other antigens. Eight patients had negative skin tests to C. albicans, but reacted to other antigens. Four patients were negative to all antigens in the panel and anergy was confirmed by their failure to become sensitized by topical application of 1-chloro-2,4-dinitrobenzene. One of these patients has an immunological profile similar to the Nezelof syndrome.

The experiments with cultured lymphocytes have disclosed further heterogeneity of the patients' immune responses. No patient with negative skin reactions produced MIF in vitro. Ten of the 12 skin test negative subjects did not show antigen-induced lymphocyte proliferation in vitro, however, in 2 patients these responses were normal.

Studies with PHA in lymphocyte cultures have also demonstrated heterogeneity of responses. Anergic patients generally have impaired PHA responses, while those patients in whom only the response to Candida was impaired had normal responses.

Proposed Course:

These studies will be extended to include additional subjects and to investigate the possible role of serum factors in the aberration of the PHA response.

Significance to Biomedical Research and the Program of the Institute:

Data presented from this laboratory have shown that many patients with chronic mucocutaneous candidiasis have abnormalities of cellular immunity. Furthermore, the abnormalities are not the same from patient to patient. The observations presented above have been synthesized into a model for the pathogenesis of the disease which is described later.

Objectives (Sub-project B)

1. Development of a program for treatment of chronic mucocutaneous candidiasis based on clinical and immunological features.

Methods Employed:

In our series of patients, candidiasis either began between infancy and the third year of life, or between the ages of 8-13 years. Those patients with "late-onset" disease were frequently under treatment with antibiotics for disorders such as acne vulgaris' or furunculosis at the time of development of candidiasis.

We have proposed that patients with early onset disease have a defect in cellular immunity that has a congenital basis. In these patients, therapy must be directed at both eradication of the organism and restoration of intact cellular immunity. Patients with "late-onset" candidiasis may have a defect in host-defenses, but it has not been defined. From the results of the therapeutic trials, we believe that the immunologic abnormalities observed in this group of patients are acquired.

Amphotericin B has been used as the antifungal agent. The dosage range has been small, 800-1100 mgm, and administered over 7-8 weeks. When cultures from the nail folds become negative, the nails are avulsed in the belief that this is one of the reservoirs for recurrent infections.

Immunologic reconstitution has been done with transfer factor, a dialyzable substance obtained by disrupting leukocytes from skin test positive subjects. The optimal protocol for transfer factor is unknown, however, our protocol has been monthly injections for 3 months, then every other month. Recipients are monitored from in vivo skin responses and in vitro lymphocyte responses.

Major Findings:

Three patients with late onset candidiasis were treated with amphotericin B and nail avulsion. All three patients have remained free of disease for 2 1/2, 1 1/2 and 8 1/2 years respectively. The two patients who were candida skin test negative have converted to positive skin tests.

Five patients have been treated with transfer factor alone. Although skin test reactivity and MIF production were observed in four of the patients, there were no clinical responses.

Two patients have been treated with the combined program of amphotericin B and transfer factor. Complete clearing of the mucocutaneous lesions occurred and both patients have been in remission for over seven months.

Proposed Course:

We plan to extend these treatment schedules to additional patients and continue the follow-up studies on the subjects who have been treated.

Significance to Biomedical Research and the Program of the Institute:

From this experience it seems reasonable to assume that consideration of both clinical and immunological factors is essential for selection of effective therapy in candidiasis. Patients with "late-onset" disease may or may not have defective cellular immunity. The defect in these patients is apparently acquired and analogous to "immune-deviation" in experimental animals. Clearing of the fungal infection with amphotericin also reduces the tolerogenic load of antigen. In two cases the patients have become responsive to the antigens of candida, a event that would be expected in view of their obvious exposure to the antigen.

The studies with anergic patients with candidiasis since early childhood suggest that combined antibiotic-reconstitutive therapy may induce long remissions. It also appears that "transfer factor" alone is not adequate for induction of remissions. However, it must be recognized that the dose or schedule of therapy with transfer factor is unknown.

Ojectives (Sub-project C)

1. Mechanism of action of transfer factor.

2. Therapeutic trials with transfer factor in patients with defined immune deficiency syndromes.

Method Employed:

The in vivo and in vitro tests of immunologic functions have been described in sub-project A.

In addition, three patients with Nezelof syndrome have received transfer factor (in collaboration with Dr. Lauren Pachman). This disease is apparently due to a defect in the thymus dependent immune system while the serological responses are intact.

Major Findings:

Reconstitution of immune functions in candidiasis as indicated by conversion of negative skin tests to positive has occurred in 4 of 7 recipients of transfer factor. The failures were in patients with Nezelof's syndrome and will be discussed in more detail below. There has been good correlation between skin test reactivity and MIF production by cultured lymphocytes. In contrast, restoration of lymphocyte transformation responses has occurred only once.

Proposed Course:

These studies will be extended to additional patients. Preliminary experiments on establishment of an animal model for transfer factor have been started.

Significance to Biomedical Research and the Program of the Institute:

These observations may be interpreted within the framework of the model for delayed hypersensitivity presented from this laboratory. The model envisions a multicellular system for antigen recognition and lymphokine synthesis analogous to that demonstrated for certain antibody responses in rodents. In the model, both antigen reactive cells and lymphokine producing cells would be "thymus-dependent".

The model therefore predicted that athymic patients with the Nezelof syndrome would not respond to transfer factor and this prediction has been substantiated in 3 cases.

The responses to transfer factor in the candidiasis patients may indicate that their immunological defect lies at a site that prevents expression of cellular immunity by lymphokine production. The lesion is apparently corrected or circumvented by transfer factor. Obviously, future experiments will be addressed to more precise delineation of the defect and the mechanism of action of transfer factor.

Objectives (Sub-project D):

1. Search for linkage between chronic mucocutaneous candidiasis and histocompatibility antigens.

Method Employed:

Peripheral blood lymphocytes have been typed for histocompatibility antigens by the microcytotoxicity assay. This work was done under a contact with Dr. Paul Terasaki, UCLA Medical School.

Major Findings:

Several major HL-A antigens occur in abnormal frequencies in candidiasis patients. The frequencies of HL-A 1, 2, 9 and 10 at the first sub-locus and 8 and 12 at the second sub-locus are less than normal while HL-A occurs twice as often as in the control group.

Proposed Course:

These studies will be extended to include new patients as well as other patients with immune defects.

Significance to Biomedical Research and the Program of the Institute:

Studies in animals have shown that responsiveness to certain antigens is genetically controlled and linked to major histocompatibility antigens. Subsequently, human studies have shown unusual frequencies of HL-A antigens in patients with SLE, leukemia, Hodgkins disease and non-tropical sprue.

The finding of an unusual frequency of HL-A in patients with candidiasis may be evidence for linkage of immunologic unresponsiveness in these patients to a histocompatibility antigen. These studies will be pursued to clarify this point.

Objectives (Sub-project E):

1. Characterization of serum antibody specificities in chronic mucocutaneous candidiasis.

2. Screening of patients sera for free candida antigens.

Method Employed:

Over 50 antigenic specificities have been identified in rabbit antisera to disrupted C. albicans. In collaboration with Dr. Nils Axelsen, the scientist who has identified these antigens, we have screened sera of candidiasis patients for their antibody profiles. The technique uses crossed antigen-antibody electrophoresis and gives both qualitative and quantitative data.

By using patients serum in place of the candida fractions, the system has been adapted for detection of free antigens in serum.

Major Findings:

The serological profiles of six patients have been completed and six additional patients are under study. The qualitative and quantitative responses divided the patients into 2 groups. One group had antibodies against many antigenic fractions and the titers were generally very high. The second group had few responses and low titers. Of particular interest was the fact that the patients with numerous and generally high-titered responses were those with the most extensive and severe cutaneous disease. Free candida antigens have been identified on only one patients.

Proposed Course:

These studies will be extended to the new candidiasis patients as they become available. Tissue studies of antigen, antibody and C are planned.

Significance to Biomedical Research and the Program of the Institute:

The mechanisms of the extensive cutaneous inflammation in candidiasis patients is unknown. The defective cellular immunity makes this route of inflammation unlikely. However, the marked serological responses in these patients may indicate a role for antigen-antibody-complement complexes in the pathogenesis of this form of candidiasis. This postulate is supported by the finding of free candida antigens in the serum from one patient. The alternative explanation for the serologic findings is that these responses are secondary to chronic intradermal immunization with candida.

Confirmation of these models will be difficult but immunofluorscent studies of skin biopsies are planned.

Objectives (Sub-project F)

1. Characterization of allergy to commercial thyrotropin in a patient with candidiasis and hypoparathyroidism.

Method Employed:

A patient with candidiasis and hypothyroidism had an immediate-type allergic reaction following injection of thyrotropin for a routine test. Antibody assays were done by gel diffusion and skin testing using standard commercial thyrotropin and purified preparations of proteins shown to contaminate thyrotropin.

The effect of the patient's serum and control sera on the biological activity of standard thyrotropins was also measured. These studies were conducted in collaboration with Dr. J. C. Meek.

Major Findings:

The immunological studies indicated that bovine serum albumin (BSA) contaminating the TSH was the antigen responsible for the reaction. The patients serum did not neutralize standard TSH.

Proposed Course:

This project has been completed.

Significance to Biomedical Research and the Program of the Institute:

For years it has been known that many normal people have antibodies to bovine proteins. These antibodies have been considered as benign concommitants of ingestion of beef products. This patient showed a point that has generally been ignored, that is, under certain circumstances these antibodies may be of pathologic importance.

Patients with chronic candidiasis often have abnormalities of endocrine function. The pathogenesis is unknown. The studies of serum-TSH interactions were undertaken in an effort to elucidate a mechanism for hypothyroidism in our patient.

Objectives (Sub-project G)

1. Identification of the antigen-sensitive cell responsible for this graft-vs-host reaction (GVH).

2. Development of method of selectively removing the GVH cells.

Methods Employed:

It has been shown that incubation of replicating cells with bromodeoxyuridine followed by exposure to light will selectively kill cells dividing in response to certain antigens. The surviving cells are capable of responding to other antigens.

This method has been used to study cells participating in mixed leukocyte reactions and graft versus host reactions.

Major Findings:

The proper schedule and dosage of BUdR and light treatment would reduce thymidine incorporation by cells in mixed leukocyte reactions by 80%. The same cells were reduced in their capacity to elicit local graft-versus-host reaction by 80%.

Proposed Course:

Studies of MLC and GVH in monkeys have been started in order to evaluate the effect of BUdR and light on transplantation in primates.

Honors and Awards:

1. Extra-laboratory activities of the Principal Investigator -

   a) Chairman, NIAID Clinical Research Committee

   b) Member, Medical Board, NIH, 1970-72

   c) Project Officer, NIAID Contract with Allergy Foundation of America to establish criteria for diagnosis of allergic diseases

   d) Project Officer, NIAID Contract for purification of antigens E, Ra3 and K from short ragweed pollen

   e) Member, Special Study Section, NIAID Allergy Clinical Center, 1972

   f) Member, Special Study Section, NHLI, Specialized Center for Research (SCOR) for pulmonary disease

   g) Member, Weather and Air Pollution Committee and Audio-visual Committee. American Academy of Allergy, 1972

Publications:

1. Green, I., Kirkpatrick, C. H. and Dale, D. C.: Lactoferrin-specific localization in the nuclei of human polymorphonuclear neutrophilic leukocytes. Proc. Soc. Exp. Biol. Med. 137: 1311-1317, 1971.

2. Kirkpatrick, C. H., Green, I., Rich, R. R. and Schade, A. L.: Inhibition of growth of Candida albicans by iron-unsaturated lactoferrin: Relationship to host-defense mechanisms in chronic mucocutaneous candidiasis. J. Inf. Dis. 124: 539-544, 1971.

3. Johnson, R. A., Smith, T. K. and Kirkpatrick, C. H.: Agmentation of phytohemagglutinin mitogenic activity by erythrocyte membranes. Cell Immunol. 3: 186-197, 1972.

4. Rich, R. R., Kirkpatrick, C. H. and Rosenthal, A. S.: Photosensitive cheilitis: Clinical and pathogenetic considerations. Ann. Int. Med. 75: 909-917, 1971.

5. Kirkpatrick, C. H., Rich, R. R., Graw, R. G., Jr., Smith, T. K., Mickenberg, I. D., and Rogentine, G. N.: Treatment of mucocutaneous moniliasis by immunologic reconstitution. Clin. Exp. Immun. 9: 733-748, 1971.

6. Kirkpatrick, C. H., Rich, R. R., and Bennett, J. E.: Chronic mucocutaneous candidiasis: Model building in cellular immunity. Ann. Intern. Med. 74: 955-978, 1971.

7. Johnson, R. A., Baer, H., Kirkpatrick, C. H., Dawson, C. R. and Khurana, R. G.: Comparison of contact allergenicity in humans of the four pentadecyl catechols derived from poison ivy urushiol. J. Allergy, 49: 27-35, 1972.

8. Kirkpatrick, C. H., Lunde, M. N. and Diamond, L. S.: Cutaneous responses of normal subjects of histolyticin from Entameba Histolytica. Am. Trop. Med. and Hyg., 21: 18-21, 1972.

Serial No. NIAID-36(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Structural Correlates of Antigen and Mitogen Interactions with Immunocompetent Cells

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. D. L. Rosenstreich
Mr. J. Thomas Blake

Cooperating Units: Dr. J. M. Davie, LI, NIAID, NIH (Project No. 140)
Dr. J. Johnson, Scripps Clinic and Research Foundation, La Jolla, California
Dr. William Paul, LI, NIAID, NIH (Project No. 140)
Dr. Jack Stobo, LI, NIAID, NIH (Project No. 141)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1-3/12			1-3/12
Professional:	9/12			9/12
Other	6/12			6/12

Project Description:

Objectives:

1) To develop general and specific cytochemical techniques which would permit visualization of antigen at the light and electron microscope level. Electrophoretically pure horseradish peroxidase and related peroxidases have been chosen for study because they will potentially permit both light and electron microscope study.

2) To correlate at a morphologic level cell interaction and the immunologic responses of such a model system.

3) To determine the cellular fate of antigen or mitogenic proteins in in vitro and in vivo models of cellular and humoral immunity using visible markers.

Methods Employed:

Electrophoretically pure horseradish peroxidase and cytochrome C peroxidase have been chosen as antigens with potential as visible immunologic

markers. The following techniques have been applied to permit characterization of the immunobiology of these proteins as antigens and record the factors determining their use as detectable antigens at a cytochemical level:

1) Deoxyribonucleic acid synthetic response of lymph node, spleen, thymus and peripheral blood cells.

2) Nature and characterization of the antibody synthetic response in vitro and in vivo. A technique for simultaneous immunodiffusion studies and cytochemistry has been developed.

3) Isolation of anti-HRPO binding material from guinea pig serum by selective immune absorbent column chromatography and subsequent purification over Sepharose G-200 into Ig subclasses.

4) Specific antisera were raised against the Ig and anti-HRPO antibody and were characterized by Farr precipitation assays and hemagglutination studies performed by established and published techniques.

5) Preparation of antigenically active but enzymatically inactive derivatives are prepared by acid-acetone extraction of the heme group at -40°C, the heme moiety being required for enzymatic activity of the peroxidatic protein.

6) Horseradish peroxidase (HRPO) can be coupled to other immunogenic proteins by using gluteraldehyde as a cross-linking reagent at pH 7.0 and the derivative purified by starch block electrophoresis.

7) Antigen binding studies: Teased lymph node or spleen cells are washed x 3 in 1% serum-suspension media (Eagle's) and at a concentration of 100-200 million per milliliter incubated with or without antigen at various concentrations and at 4°C for 30-60 minutes. These cells are then washed 3-4 times over 100% fetal calf serum by density gradient and resuspended in 0.5 ml pure fetal calf serum and smeared on glass slides. Cells are air dried and fixed in either gluteraldehyde, methanol (-40°C) or acid-acetone (-40°C-70°C). After washing away fixative some slides are incubated with horseradish peroxidase and fixed in gluteraldehyde. Slides are then incubated in 3,3-diaminobenzydine in tris-HCl buffer at pH 7.6 and reaction product visualized after mordinification and dehydration for light microscopy.

Alternatively aliquots of gluteraldehyde fixed cells were processed for electron microscopy.

Major Findings:

1) Immunoglobulin and thus presumably antigen specific receptors exist tightly bound to the surface membrane of B lymphocytes. T lymphocytes by contrast lack easily detectable surface immunoglobulin. On interaction with antigen or antibodies directed against these determinants, migration of these receptors towards the centrosome associated cell pole occurs followed by

internalization. While binding to these receptors occurs at 0-4°C, migration in contact with a multivalent ligand requires expenditure of cell energy for "capping" or internalization. However a clumping or aggregation of these prescribed receptors occurs within the plane of the cell membrane even in the presence of metabolic inhibitors. We have visualized at the ultrastructural level this phenomenon using a rabbit anti-guinea pig immunoglobulin reagent coupled to horseradish peroxidase (RAGIG-HRPO). Studies of the fate of internalized receptor with time is being pursued. (Work in collaboration with Drs. J. Davie and W. Paul).

2) In collaboration with Drs. J. Stobo and W. Paul we have investigated the interaction and fate of concanavalin A, a plant lymphocyte phytomitogen, with the target cell. EM studies as well as biochemical studies show a complex activation event both in terms of kinetics and differential responsiveness to Con A and PHA indicating heterogeneity of T-lymphocyte. Using Con-A-Cytochrome-C peroxidase complexes Con A was localized to patches of the cell surface.

Significance to Biomedical Research and the Program of the Institute:

A knowledge of structural correlates of the cellular events in immunologic interactions is necessary to fully comprehend such basic phenomena as transplantation rejection and immunoglobulin synthesis. Such an understanding in these areas would contribute greatly to the likelihood of therapeutic and pharmacologic manipulation of crucial host defense mechanisms. Macrophage migration appears clearly to be an in vitro correlate of cellular immunity and as such provides a model for studies of the ultrastructure and cellular correlation of delayed hypersensitivity.

Proposed Course:

1) Attempts to synthesize HRPO conjugates with mitogen (Concanavalin A) with Drs. J. Stobo and W. Paul of the Laboratory of Immunology will be continued. After initial characterization of the biochemical and immunologic properties of these compounds the localization of mitogen receptors and their fate in the in vitro proliferation response will be studied.

2) In collaboration with Drs. J. Davie and W. Paul of the Laboratory of Immunology, we will synthesize conjugates of rabbit anti-guinea pig immunoglobulin. Once synthesized the immunoglobulin membrane surface on guinea pig lymphocyte receptors will be mapped and characterized as to long term fate in vitro.

3) The fate of antigen binding cells using conjugates of HRPO and dinitrophenyl guinea pig albumin (DNP-GPA) or radioactive DNP-GPA will be characterized and their light and ultrastructure explored.

a) Determine the kinetics of the appearance of antigen binding cells (ABC) and antibody forming cells (AFC) and explore the localization of antigen at the level of the electron microscope.

b) Quantitation of receptors per cell and its relationship to the immunocompetence of the animal.

c) Attempt to differentiate antibody forming cell precursor from antibody binding cells and their origin (thymic or marrow).

Honors and Awards: NONE

Publications:

1. Davie, J.M., Rosenthal, A.S., and Paul, W.E.: Receptors on immunocompetent cells. III. Specificity and nature of receptors on ^{125}I-DNP-GPA antigen binding cells of immunized guinea pigs. J. Exp. Med. 134: 517-531, 1971.

2. Stobo, J., Rosenthal, A.S., and Paul, W.E.: Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of Concanavalin A and phytohemagglutinin. J. Immun. 108: 1-17, 1972.

3. Stobo, J., and Rosenthal, A.S.: Biologically active Concanavalin A complexes suitable for light and electron microscopy. Exp. Cell Res. 70: 443-446, 1972.

Serial No. NIAID-40(c)
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Clinical Studies on Patients with Known or Suspected Parasitic Diseases

Previous Serial Number: SAME

Principal Investigators: Dr. Franklin A. Neva
Dr. Peter T. Franck
Dr. Theodore E. Nash

Other Investigator: Dr. Michael M. Frank

Cooperating Units: Dr. David W. Alling, OSD, NIAID (No project number)
Dr. Allen W. Cheever, LPD, NIAID (Project number 121-H)
Dr. Jerry Dahl, Chief of Medical Operations, Peace Corps., Washington, D. C.
Dr. Richard Glew, LPD, NIAID (Project number 134)
Dr. William Howard, LPD, NIAID (Project number 134)
Dr. Myron Schultz, Parasitic Disease Drug Service, National Center for Disease Control, Atlanta, Georgia

Man Years:

	Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2			2
Professional:	1-9/12			1-9/12
Other:	3/12			3/12

Project Description:

Objectives:

1. Study pathophysiology of parasitic diseases.

2. Develop improved criteria to assess severity or extent of disease, indications for treatment, and reversibility of disease process.

3. Contribute to training of clinical associates, guest workers, and medical community in general concerning clinical diagnosis and management of parasitic diseases.

Methods Employed:

Clinical contacts with various medical facilities continue to be made to establish our interest in clinical parasitology. Increasing numbers of

patients, many of them NIH employees, are being seen (mainly in the OPD, but occasionally in hospital) for evaluation of illness or symptoms after recent return from tropical environments.

The schistosomiasis patients are being studied immunologically for circulating antigen and antibody with gel diffusion and immunoelectrophoresis procedures with reagents prepared from infected animals. Standard blood smears and methods for determination of complement levels are being used for study of malaria patients.

Major Findings:

Most of the schistosomiasis cases that we have studied have not exhibited definite morphologic or functional abnormalities attributable to infection. Two of the patients showed presence of antibody to the circulating antigen that is being studied by Dr. Nash. We have been impressed by the frequent observation of white cell clustering to S. hematobrium eggs. Several patients with cutaneous leishmaniasis were studied in the past year--one with nasal mucous membrane involvement whose infection was probably acquired in Spain. Considerable effort was expended in attempting to determine the etiology of choreoretinitis in two girls from New Jersey whose illnesses suggested a common source infection, but even a field epidemiologic investigation by CDC failed to reveal etiologic dues. Another patient who defied specific diagnosis, and who may represent a type of problem to be encountered increasingly in the future was a Vietnam veteran suspected of having a gnathostome infection. Collaborative efforts by Dr. Michael Frank of LCI have shown that the transient fall in levels of whole serum complement seen in some of our malaria patients is also reflected in a fall in C4 levels.

Significance to Biomedical Research and the Program of the Institutes:

The research and service activities of this Section complements the various disciplines within LCI and helps provide a well-balanced training and research program in infectious diseases. It is planned that there will be an increasing collaboration of some of the malaria studies in LPD with Dr. Frank's group in LCI on the mechanism and significance of the alterations in complement during the malarial paroxysm. The schistosomiasis work of Dr. Nash also emphasizes immunologic aspects of parasitic infection.

Proposed Course: To be continued as planned

Honors and Awards:

Dr. Theodore E. Nash -
Consultant - to Pan American Health Organization in Brazil from December 19, 1971 to January 25, 1972.

Dr. Peter T. Franck -
Participant - invited to participate in a Pan American Health Organization Symposium on Venezuelan Equine Encephalitis held in Washington, D.C., Sept.,1971.

Publications: NONE

Serial No. NIAID-44(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Pathophysiology of Autoimmune Hemolytic Anemia

Previous Serial Number: SAME

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Michael Kane
Dr. John Atkinson

Cooperating Units: Dr. Richard Root, University of Pennsylvania, Philadelphia, Pennsylvania
Dr. Alan Schreiber, Robert Breck Brigham Hospital, Boston, Massachusetts

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1			1
Professional:	11/12			11/12
Other:	1/12			1/12

Project Description:

Objectives:

1. To define the pathophysiologic function of antibody and complement on the surface of red cells in patients with acute hemolytic anemia and to determine the effect of these substances on red cell survival. To determine how steroid therapy and splenectomy influences the survival of erythrocytes sensitized with antibody and complement. To this end a model of immune clearance of erythrocytes has been established in the guinea pig.

Methods Employed:

Rabbits have been immunized with guinea pig erythrocytes utilizing a number of different routes of injection, and IgM and IgG immunoglobulin fractions of high purity have been prepared from the rabbit antisera. Fractions containing antibody with high avidity for guinea pig erythrocytes have been used to sensitize guinea pig erythrocytes. The number of complement fixing sites per erythrocyte formed by these antibodies has been determined by use of the C1a fixation and transfer test. The antibodies have been used to sensitize guinea pig erythrocytes labeled with chromium 51 and the half life of the cells

studied after reinjection into guinea pigs. In some experiments the immunoglobulin fractions have been radiolabeled with ^{125}I by the Iodine Monochloride method and the absolute number of molecules of antibody on the cell surface determined by radioactive I uptake. Clearance studies have been performed in splenectomized and cortisone treated animals.

Major Findings:

Our observations indicate that the half-life of chromated erythrocytes in the guinea pig is seven and one-half days. We find that sixty complement fixing IgM sites per cell are required before the cells show increased rates of clearance. When cells with sixty or more IgM sites are injected into the guinea pig, they are immediately cleared by the liver and then re-released into the circulation where they undergo a normal survival. The situation with respect to IgG sensitized cells is entirely different. As few as 1.4 complement fixing sites per erythrocyte are sufficient to lead to decreased survival of erythrocytes. These cells are not sequestered in the liver within five minutes of injection but undergo a straight-line, decreased survival curve for many hours with sequestration in the spleen. They are not re-released into the circulation. A small proportion of the IgG-coated cells do show normal subsequent survival after a period of several hours. That complement is important in the clearance of these cells has been shown by parallel studies in our newly described strain of guinea pigs with the total, genetically controlled deficiency of C4. In these animals IgM sensitization produces no sequestration of the cells and the cells undergo a normal survival. In the case of IgG, there is slightly decreased survival in C4 deficient animals of cells with a very large number of IgG molecules per cell, however, this does not approximate the rate of clearance noted in animals with intact complement function.

Splenectomized animals clear IgM coated cells normally. They have a marked defect in their ability to clear IgG coated cells, however, over a period of time, the liver develops the capacity to partially clear the IgG coated cells. Cortisone treated animals are deficient in their ability to clear both IgG and IgM coated cells.

Significance to Biomedical Research and the Program of the Institute:

These studies define the role of antibody and complement in clearance of RBC's in patients with the disease, autoimmune hemolytic anemia. They represent the first molecular studies of the effect of immunoglobulin and complement on the survival of erythrocytes. They explain the finding, known for many years, that patients with hemolytic anemia may have Coombs positive cells in their circulation, which have a perfectly normal survival. Presumably, the cells have complement components placed on their surface by IgM. The studies point out the *in vivo* importance of earlier studies from this laboratory, demonstrating that IgM and IgG handle complement components differently and produce different kinds and amounts of damage to cell surfaces. The studies provide an explanation for the finding that some patients with hemolytic anemia respond to splenectomy and others do not and provide an explanation of the therapeutic response of patients to treatment with cortisone.

Proposed Course:

The initial studies have been completed and have been published. The studies of cortisone effect and the effect of splenectomy on clearance rates are in progress. Approval has been obtained to extend these studies to human beings and to determine whether the factors which influence survival of erythrocytes in animals are the same as those which influence survival in humans.

Honors and Awards:

An abstract of this work was selected for presentation at the American Hematology Society Meeting, December, 1971.

Publications:

1. Schreiber, A. D. and Frank, M. M.: Role of antibody and complement in the immune clearance and destruction of erythrocytes. II. Molecular nature of IgG and IgM complement-fixing sites and effects of their interaction with serum. J. Clin. Invest. 51: 583-589, 1972.

2. Schreiber, A. D. and Frank, M. M.: Role of antibody and complement in the immune clearance and destruction of erythrocytes. I. In vivo effects of IgG and IgM complement-fixing sites. J. Clin. Invest. 51: 575-582, 1972.

Serial No. NIAID-45(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ultrastructural Correlates of the Cellular Events in Host Defense Mechanisms in Man and Experimental Animals

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. David L. Rosenstreich
Mr. J. Thomas Blake
Dr. Sheldon M. Wolff
Dr. Harry R. Kimball
Dr. Charles H. Kirkpatrick
Dr. Burton C. West
Dr. Michael M. Frank
Dr. Ethan M. Shevach

Cooperating Units: Dr. David C. Dale, Univ. of Washington Hospital, Dept. of Medicine, Seattle, Washington
Dr. Anthony S. Fauci, The New York Hospital-Cornell Medical Center, Dept. of Medicine, New York, New York
Dr. John Finerty, LMI, NIAID, NIH (No project no.)
Dr. Ira Green, LI, NIAID, NIH (No project no.)
Dr. John Martin, LI, NIAID (No project no.)
Dr. Robert R. Rich, Harvard Medical School, Dept. of Pathology, Boston, Massachusetts
Dr. Richard K. Root, Univ. of Pennsylvania School of Medicine, Dept. of Medicine, Infectious Disease Section, Philadelphia, Pennsylvania
Dr. Robert E. Scott, University of Minnesota School of Medicine, Dept. of Pathology, Minneapolis, Minnesota
Dr. John van Boxel, LI, NIAID, NIH (No project no.)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	3-3/12			3-3/12
Professional:	2-11/12			2-11/12
Other:	5/12			5/12

Project Description:

Objectives:

1. Provide ultrastructural analysis of the cellular events in clinical disorders of the host differences in man and experimental animals.

2. Develop new morphologic techniques for investigating animal models of human disease.

3. Provide support for other investigators in their clinical and experimental approaches to human disease.

Methods Employed:

Methodologic approaches are as varied as the disorders to which they have been applied but in general involve fixation of tissue specimens or material provided by the collaborating units. These specimens are subjected to autoradiographic, cytochemical, ultrastructural and morphometric analysis by standard techniques. Where specific methodologies have been specifically developed, they will be briefly described under subject headings reviewed under Major Findings.

Major Findings:

1. Drs. David Dale, Robert Scott and Sheldon Wolff - Ultrastructural analysis of bone marrow and peripheral blood of cyclic neutropenia in grey collies.

A defect of cell maturation has been detected at the stage of 2nd granule formation in the developing marrow neutrophil. The appearance of such a lesion corresponds to the cyclic fluctuation of marrow differentiation in the grey collie. This defect, cytoplasmic autophage, glycogen aggregate deposits, accumulation of membrane vesicles suggest an autoregulatory defect of genetic origin in these dogs.

2. Dr. Burton West and Dr. Harry Kimball - Isolation of human neutrophil granules by sucrose isopyicnic gradients.

A biochemical analysis of gradient separated granules has been subjected to a corresponding electron microscopic analysis. Micrographs of granule fractions correlated morphologically with segregation of enzymatic activity into distinct classes. Interestingly, alkaline phosphatase activity in human WBC appears to be associated not with a granule but with a cell membrane fraction.

3. Dr. Robert Rich and Dr. Charles Kirkpatrick - Photosensitive chelitis.

We have described a case of u.v. light induced chelitis associated with ultrastructural evidence of nuclear bodies and cytoplasmic smooth endoplasmic reticulum particles resembling the myxovirus like particle seen in systemic lupus erythematous glomeruli by electron microscopy. These lesions were reversed by u.v. lights.

4. Dr. Richard Root - Ultrastructural correlate of impaired granulocyte bactericidal activity in Chediak-Higashi Disease (CHD).

We have characterized by electron microscopy the impaired killing of

bacterial by CHD granules and shown that the abnormal CHD granules exhibit delayed fusion with phagosomes. By use of peroxidase cytochemical techniques we can show that myeloperoxidase, an enzyme necessary for bactericidal activity, through an halogenation mechanism is retained within the abnormal granules and is not released in a normal fashion.

5. Drs. Michael Frank, Ira Green and John van Boxel - Does cytotoxic killing involve complement?

Red blood membranes were prepared and examined for complement specific lesion or "holes". Studies as yet incomplete have failed to identify such lesions but immunologic data do suggest a specific involvement of γ_2 immunoglobulin.

6. Drs. John Finerty and John Martin - A technique for the isolation of infectious plasmodia bergeii free of host red blood cell membranes.

P. Bergeii infected mouse red blood cells were exposed to Tris-NH_4CL lysis and the biologic properties of the isolated P. Bergeii studies. Electron micrographic analysis demonstrated that the infectious organisms are undamaged and free of host membrane. Immunization with such preparation yields cytotoxic P. Bergeii specific antibodies. This represents the first such isolation procedures free of containing host material.

7. Drs. Anthony Fauci and Sheldon Wolff - The ultrastructure of the kidney of Wegener's Granulomatosis.

Immune complexes have been observed in 2 cases of Wegener's granulomatosis. Focal glomerular lesions are also present in the remaining cases examined ultrastructually. A correlation in the clinical severity of the disease process and the degree of functional renal involvement with the EM findings was observed.

Significance to Biomedical Research and the Program of the Institute:

1. Complete studies in progress.

2. Expand areas of promise commenserate with the available material.

3. Suggest from electron microscopic data new biologic approaches to problems already under study.

Honors and Awards: NONE

Publications:

1. Martin, W.J., Finerty, J., and Rosenthal, A.S.: Isolation of plasmodium berghei (Malaria) parasites by ammonium chloride lysis of infected erythrocytes. Nature NB 233: 260-261, 1971.

2. Song, C.S., Moses, H.L., Rosenthal, A.S. Gelb, N.A., and Kappas, A.: The influence of postnatal development on allylisopropyl acetamide induced hepatic porphria and synthesis of cytochrome P-450 in rats: A biochemical and morphological study. J. Exp. Med. 134: 1349-1371, 1971.

3. McGee, Z. A., Ratner, H.B., Bryant, R.E., Rosenthal, A.S., and Koenig, M.E.: Host defense against L-phase variants: An antibody complement system in human serum lethal to L-phase variants of bacteria. J. Infect. Dis. 125: 281-282, 1971.

4. Root, R.K., Rosenthal, A.S., and Balestra, D.J.: Abnormal bactericidal, metabolic and lysosomal function of Chediak-Higashi syndrome leukocytes. J. Clin. Invest. 51: 649-665, 1972.

Serial No. NIAID-46(c)
1. LCI
2. Biologic Structure Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunobiologic and Ultrastructural Characterization of Cellular Immune Effector Cells.

Previous Serial Number: SAME

Principal Investigator: Dr. Alan S. Rosenthal

Other Investigators: Dr. David L. Rosenstreich
Mr. J. Thomas Blake
Dr. Peter E. Lipsky
Dr. James E. Balow

Cooperating Units: NONE

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	2			3
Professional	1-7/12		1	2-7/12
Other:	5/12			5/12

Project Description:

Objectives:

1) To develop and apply techniques for the isolation of cellular immune effector cells.

2) To characterize in a quantitative and qualitative fashion the response of such cells to a variety of in vitro correlates of cellular immunity such as antigen-dependent lymphoycte proliferation and macrophage inhibitory factor production.

3) To characterize the ultrastructures of cellular immune effector cells as contrasted with other types of lymphocytes.

4) To study the mechanisms of lymphoycte activation by antigen and mitogen using visible antigenic markers such as horseradish peroxidase (HRPO) and HRPO-protein conjugates.

5) To study the regulation of cellular events and the role of cell cooperation and its relationships to humoral immunity.

6) Characterize the role of macrophages in cell activation.

Methods Employed:

1) Determination of DNA synthesis of lymphocytic populations with and without addition of antigen by 4 hour pulse of ^{3}H-TdR incorporation. Results assayed by either/or both autoradiography or liquid scintillation spectrometry

2) Preparation and isolation of partially purified population of lymphocytes from oil induced peritoneal exudates or regional draining nodes subsequent (3-4 weeks) to immunization of inbred strain 2 and 13 guinea pigs with chemically defined antigens such as horse spleen ferritin, horseradish peroxidase or hapten protein conjugates.

3) Column purification: Peritoneal exudate cells (PEC) or lymph node cell suspensions were passed over glass bead columns and incubated at 37°C for 1 hour. Cells were then eluted with 10% guinea pig sera in minimal essential Eagle's media and the recovered cells studied.

4) Ultrastructural analysis of antigen localization by means of autoradiography (isotopically labelled antigen) or cytochemistry (peroxidase coupled or direct use).

Major Findings:

1) Complex lymphoid cell mixture placed over glass bead columns are depleted of antibody forming cells and their precursors as measured by loss of plaque forming cells (PFC'S); immunoglobulin and antigen specific receptor bearing cells as measured autoradiographically. By contrast those cells which participate in in vitro correlates of cellular immunity such as antigen-dependent lymphoycte proliferation and elaboration of macrophage inhibitory factor (MIF) are excluded from the column.

2) There appears to be within the lymphoreticular system a segregation both functionally and anatomically by immune competence. Those cells which mediate cellular immune function appear to be sequentered within inflammatory sites such as oil-induced peritoneal exudates. These sequestered cells possess enhanced immune responsiveness producing greater inhibition of macrophage migration by a factor of 10 than an equivalent number of lymphocytes derived from the region nodes. Moreover, antigen recognition as indicated by new DNA synthesis in inflammatory exudate lymphocytes is 30-200 fold greater than an equivalent number of lymph node lymphocytes. These differences can not be explained by presence or absence of macrophages, significant blastogenic factor release or desparities in the kinetics of cell responsiveness.

3) The peritoneal exudate lymphocyte have unique surface membrane changes on ultrastructural analysis.

4) The lymphocytes within the peritoneal exudate (PELS) appear to represent thymus-derived or T-lymphocytes. At least two types exist.

One possesses a cell surface membrane modification, a uropod, whose formation is metabolically dependent. B lymphocytes which bear surface immunoglobulin do not appear to form uropods.

5) Activation of immunocompetent T lymphocytes is a complex phenomenon. While the exact mechanism is not established our data strongly indicate a multi-step event in which an initial interaction of antigen with an immunologically naive macrophage occurs followed by T-cell macrophage interaction and thence antigen recognition. T lymphocytes do not themselves appear capable of direct interaction with soluble antigen. Regression analysis of cell density and studies employing metabolic inhibitors indicate a necessary physical interaction between the immunospecific T-lymphocyte and the macrophage being immunorelevant antigen.

Significance to Biomedical Research and the Program of the Institute:

The isolation and characterization of an enriched population of cellular immune effector cells should provide a useful system for study of the role of the cellular immune effector cells in immunologic surveillance and their contribution to the humoral limb of host defenses. Tumor immunity and transplantation immunity have been shown to rely to a great extent upon mechanisms of cellular immune function such as cytotoxicity and elaboration of other so-called "soluble mediators." A more fundamental understanding of the immunobiology of these crucial events and morphologic correlates may provide new insights into immune competance in man and information on the mechanisms of cellular regulation of the immune responsiveness. Potential approaches to therapeutic manipulation of such events would be of immediate clinical relevance.

Proposed Course:

1) Characterize the regulation of lymphocyte activation.

2) Characterize the ultrastructure of cellular immune effectors.

3) Characterize the localization and fate of antigen in in vitro antigen-mediated lymphocyte proliferation.

4) Explore the role of cellular immune effector in cell - cell interaction and cooperative activity with those cells required for antibody formation and transplantation immunity.

5) Characterize the role of the macrophage in expression of cell-mediated immunity in experimental animals and man.

Honors and Awards: NONE

Publications:

1. Rosenthal, A.S., Rosenstreich, D.L., Davie, J.M., and Blake, J.T.:

Isolation and characterization of cellular immune effector cells: Evidence for the heterogeneity of such cells in the guina pig. In O. Roy Schwarz (ed.): Proceedings of the 6th Leukocyte Culture Conference, Seattle, 1971.

Rosenstreich, D.L., Blake, J.T., and Rosenthal, A.S.: The peritoneal exudate lymphocyte. I. Differences in antigen responsiveness between peritoneal exudate and lymph node from immunized guinea pigs. J. Exp. Med. 134: 1170-1186, 1971.

Rosenthal, A.S., Davie, J.M., Rosenstreich, D.L., and Blake, J.T.: Depletion of antibody forming cells and their precursors from complex lymphoid cell population. J. Immun. 108: 279-281, 1972.

Rosenstreich, D.L., Shevach, E.M., Green, I., and Rosenthal, A.S.: Lymphocyte of the guinea pig: Evidence for thymic origin. J. Exp. Med. In press.

Serial No. NIAID-47(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Clinical Studies of Complement Abnormalities of Man

Previous Serial Number: NONE

Principal Investigator: Dr. Michael Frank

Other Investigators: Dr. Michael Kane
Dr. Robert Gerwin
Miss Thelma Gaither

Cooperating Units: Dr. J. Leddy, University of Rochester, Rochester, New York
Dr. N. Hadler, A&R, NIAMD (no project no.)
Dr. J. Decker, A&R, NIAMD (no project no.)
Dr. D. Alling, OSD, NIAID (no project no.)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1 1/12			1 10/12
Professional:	1		9/12	1 9/12
Other:	1/12			1/12

Project Description:

Objectives:

1. To define the role of complement in human illness and to develop better methods of therapy for immune damage mediated by complement.

Methods Employed:

Complement components are purified and used for estimation of component levels in human sera and spinal fluid. Double blind therapeutic studies are employed to test new methods of therapy in patients.

Major Findings:

Epsilon amino caproic acid has been shown to be an effective agent in treatment of the disease Hereditary Angioneurotic Edema and the clinical toxicity of this drug has been defined in patients suffering with this disease. A double blind study has shown that 8-12 grams/day is sufficient medication to sustain a prolonged remission. Complement component levels have been

studied in several diseases. for example, study of the blood and spinal fluid from patients with systemic Lupus Erythematosis of the central nervous system has shown that C4 can be helpful in the diagnosis of this condition but it is not absolutely diagnositc. Repeated assays are required to establish the normal CNS C4 level for each patient and deviation from normal must be compared with each patients serving as his own control. The normal levels of C4 in spinal fluid and the stability of this component in spinal fluid stored in vitro have been evaluated as part of this study.

Significance to Biomedical Research and the Program of the Institute:

EACA therapy provides a major advance in the treatment of Hereditary Angineurotic Edema. Definition of its clinical toxicity will provide physicians with some guidelines for its use in clinical situations. Similarly, studies of complement components in the cerebral spinal fluid in patients with various clinical conditions may provide insight into the pathophysiology and diagnosis of these illnesses.

Proposed Course:

These studies will be extended to other clinical conditions and complement deficiency states.

Honors and Awards: NONE

Publications:

1. Frank, M. M., Sergent, J. S., Kane, M. A. and Alling, D. W.: Epsilon amino caproic acid therapy of hereditary angioneurotic edema: A double blind study. New Eng. J. Med. 286: 808-812, 1972.

Serial No. NIAID-48(c)
1. LCI
2. Clinical Immunology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Antibody Responses in the Respiratory Tract of Rabbits Following Parenteral Immunization with Pseudomonas Aeruginosa Vaccine and Enhancement of Bacterial Phagocytosis and Killing by Respiratory Macrophages with Immune Opsonic Antibodies

Previous Serial Number: NONE

Principal Investigator: Herbert Y. Reynolds

Other Investigators: Dr. James Pennington

Cooperating Units: NONE

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1-6/12			1-6/12
Professional:	10/12			10/12
Other:	8/12			8/12

Project Description:

Objectives:

Pseudomonas aeruginosa is a serious opportunistic bacterial pathogen for patients with impaired host immunity due to hematological malignancies or immunosuppressive therapy for various neoplastic or other systemic diseases. Also, patients with extensive burns or structural lung diseases, particularly those who require assisted ventilation treatment, are at high risk to develop Pseudomonas infections. Pseudomonas colonization of the respiratory tract and subsequent pneumonia is particularly troublesome because the patient's response to antibiotics is often not dramatic and the microorganisms may not be eradicated. For example, pulmonary infections in children with cystic fibrosis are difficult to cure because antibiotics such as carbenicillin and gentamicin do not diffuse into bronchial secretions in sufficient concentrations to achieve inhibitory levels against the Pseudomonas organisms. Another approach to improve therapy of these pulmonary infections is that of immunological prophylaxis through the development of specific anti-Pseudomonas antibodies in serum and in the respiratory tract. These antibodies, particularly those with opsonic antibody activity, may help the lungs' cellular defense mechanism (pulmonary macrophages) by enhancing phagocytosis and killing of Pseudomonas.

Methods Employed:

Rabbits are vaccinated with a variety of monovalent and polyvalent whole-cell Pseudomonas vaccines to observe the development of antibody in serum and bronchial secretions. Agglutinating antibodies, total titers and 2-mercaptoethanol resistant (IgG) titers, are measured. Isolation and purification of antibodies from serum and bronchial secretions employes gel filtration and ion exchange chromatography, sucrose density gradient ultracentrifugation, zone electrophoresis and other standard concentration and separation techniques. Macrophages, established in short-term cell monolayer cultures with well described techniques, are challenged with ^{14}C labeled bacteria, which have been previously opsonized; subsequent phagocytosis is measured with liquid scintillation spectroscopy and bacterial killing is assayed with standard agar pour plate cultures. Bronchial secretion proteins are quanitated by immunodiffusion precipitation methods.

Major Findings:

Rabbits immunized parenterally for 2 weeks have serotype specific agglutinating anti-Pseudomonas antibodies in serum, limited primarily to the IgM class of immunoglobulins; whereas, bronchial secretions have no antibody activity at this time. After 4 weeks of immunization (3 injections/wk), serum antibodies are predominately in the IgG class and bronchial secretion antibody activity, present in approximately 1/4 the serum activity, is limited exclusively to the IgG class. Representative titers obtained in 12 rabbits immunized with a serotype 6 Pseudomonas strain are listed: total serum agglutination mean titer 256 (range 64-1024) with serum IgG mean titer 128 (range 64-256), corresponding bronchial mean titer 1:16 (range 4-64). Bronchial IgG antibody titers are detectable for at least 3 months after completion of immunization. The adequate development of IgG antibodies in bronchial secretions is extremely important since IgG and IgA but not IgM immunoglobulins have been identified in bronchial washings of humans and similarly in rabbits and dogs. The IgG immunoglobulins are of greatest interest because they have well described opsonic properties; although IgA concentrations are greatest in external or secretory fluids, IgA antibodies are poor opsonins which may make them less important in phagocytosis.

To evaluate the effects of specific anti-Pseudomonas antibody in bronchial secretions, phagocytic uptake and bacterial killing by respiratory macrophages in vitro were measured. ^{14}C labeled Pseudomonas organisms were opsonized with immune whole bronchial secretions or with immune IgG, IgA and IgM antibodies isolated from serum or from bronchial secretions and compared with non-immune controls. Immune bronchial secretions did not have any bactericidal antibody activity, but did promote approximately twice as much phagocytosis by macrophages as control secretions; the number of viable bacteria cultured from the immune treated macrophage monolayers were about 50 percent lower, denoting more macrophage killing. Also, IgG antibody when used as an opsonin was significantly more efficient in promoting phagocytosis and subsequent bacterial killing. Serum IgM and secretory IgA in contrast were not good opsonins and proved inferior to IgG.

Significance to Biomedical Research and the Program of the Institute:

Pseudomonas infections in patients with altered immunity or with structural lung disease is a major infectious disease problem which causes a high number of acquired infections in hospitalized patients. Recognizing that patient isolation is not feasible because of the ubiquity of the Pseudomonas organisms, which are part of the bacterial flora of many hospitalized patients, and that antibiotic therapy may be ineffective dispite the availability of good anti-Pseudomonas antibiotics, immunization of high risk patients with Pseudomonas vaccines is a reasonable addition to existing therapy.

A Pseudomonas polyvalent vaccine, developed by Parke, Davis and Co., Detroit, Michigan, is currently under investigation (DBS-IND 185). Development of this vaccine was feasible because 96 percent of the prevalent Pseudomonas aeruginosa strains could be grouped into seven immuno-serotypes, which greatly reduced the number of component antigens necessary to make a broadly protective vaccine. Immunogenic lipopolysaccharide antigens, extracted from representative Pseudomonas strains of each immunotype have been purified and combined into a heptavalent vaccine. Results obtained in a rat-burn-wound-sepsis model and following the vaccination of patients in a surgical burn unit have shown that the vaccine favorably reduces Pseudomonas wound colonization, sepsis and subsequent mortality through the development of humoral antibodies.

As yet little attention has been given to the induction of protective antibodies in the respiratory tract and to the subsequent control of pulmonary colonization by Pseudomonas. Certainly, from the experimental animal studies described, it is possible to obtain specific bronchial antibody but to do so immunization must be prolonged enough to ensure development of IgG antibody. In the presence of the antibody which is available to opsonize Pseudomonas organisms, pulmonary macrophages in vitro are more efficient in ingesting and killing these organisms.

Proposed Course:

1. Further studies will be done on the mechanism of the opsonin-bacterium-macrophage interaction to identify the active portion of the immunoglobulin antibody molecule (F_c fragment), to quantiate the amount of opsonic material needed, and to examine macrophage receptor sites for opsonic antibody.

2. Patients considered to be at high risk for Pseudomonas pulmonary infections, will be vaccinated with the Parke, Davis and Co., polyvalent Pseudomonas vaccine (Pseudogen®) to determine the optimal vaccination regimen necessary to produce specific IgG bronchial (sputum) antibodies. Observations concerning the protective value of these antibodies will be made. Further studies with human respiratory macrophages and opsonic antibodies similar to the animals experiments, will be begun.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID 49(c)
1. LCI
2. Clinical Parasitology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Laboratory Studies on Viruses of Entamoeba hystolytica and Their Possible Significance in Human Amebiasis

Previous Serial Number: NONE

Principal Investigator: Dr. Peter T. Franck

Other Investigators: NONE

Cooperating Units: Dr. Louis S. Diamond, LPD, NIAID (Project Number 122-G)

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	9/12			9/12
Professional:	6/12			6/12
Other:	3/12			3/12

Project Description:

Objectives:

1. To investigate the biology of the virus - E. hystolytica host cell relationship, in axenic as well as bacteria-associated cultures.

2. To assess the prevalence of latent viruses in E. histolytica strains obtained from patients with clinical amebiasis, in contrast with isolates from individuals with asymptomatic infection.

3. To investigate the role, if any, of viruses of E. hystolytica in the pathogenesis of human disease, either through direct effect on the human host, or by alteration of the pathogenicity of E. hystolytica.

Methods Employed:

Various of E. hystolytica are maintained in culture, either axenically or with a variety of bacterial associates. New isolates of E. hystolytica are actively sought, both from patients with disease and from symptomless carriers. The presence of virus is assayed by inoculating material from a new amebic isolate onto a battery of known ameba cell lines; conversely, new isolates are challenged with viruses known to be pathogenic for other amebic cell lines. Methods of purifying virus from cell components, virus classification by serology and host range, and cloning of amebae in an attempt to produce virus-free cell lines are under investigation.

Major Findings:

All of the established E. hystolytica cell lines now in axenic culture originated from patients with severe clinical amebiasis, and all of these lines have been found by Dr. Diamond and coworkers to contain virus. Two additional E. hystolytica isolates from patients with disease are currently under investigation. Recent evidence indicates that virus continues to be produced, and host cells remain susceptible, when grown in culture with bacterial associates, as well as in the axenic state. The nature of the virus-ameba relationship, whether "lysogenic" as in certain bacteriophage, or present in a carrier state, remains unclear. Attempts at virus purification have been rendered difficult by the fact that each E. hystolytica cell line contains its own latent virus, and no other host cell capable of supporting virus multiplication has yet been found.

Significance to Biomedical Research and the Program of the Institutes:

The problem of wide variation in the apparent pathogenicity of E. hystolytica is as old as the recognition of amebiasis as a clinical entity. The factors responsible for conversion of a symptomless carrier state to full-blown amebic disease are still unknown, and are of crucial significance to an understanding of the pathogenesis of amebiasis. Recognition of viruses of E. hystolytica isolates maintained for long periods in culture warrants investigation of their possible role in human disease.

Proposed Course:

To continue as planned, with the study of additional isolates from asymptomatic carriers as well as patients with amebic disease.

Honors and Awards: NONE

Publications: NONE

Serial No. NIAID-50(c)
1. LCI
2. Clinical Physiology Section
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanisms of Host Defense

Previous Serial Number: NONE

Principal Investigator: Dr. Joseph Bujak

Other Investigators: Dr. Harvey B. Simon
Dr. Richard D. Diamond
Dr. Ethel Patten
Dr. Burton C. West
Mr. Dominic Balestra

Cooperating Unit: NONE

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	1 4/12			1 7/12
Professional:	1 3/12		3/12	1 6/12
Other:	1/12			1/12

Project Description:

Selected patients studied in the laboratory include:

a. Patients with recurring bacterial infections including chronic granulomatous disease of childhood.

b. Patients with paroxysmal nocturnal hemoglobinuria.

c. Patients with hematologic malignancies.

d. Patients with eosinophilia.

Studies are in progress concerning the nature of defects in host defenses responsible for recurrent infections in these patients with, particular attention paid to leukocyte function.

Objectives:

Human Studies:

1. To examine the adequacy of leukocyte function in patients with recurrent infections.

2. To define the adequacy of macrophage bactericidal function in chronic granulomatous disease of childhood and thereby gain insight into mechanisms by which macrophages kill bacteria.

3. To determine if there is a neutrophil defect in paroxysmal nocturnal hemoglobinuria that helps explain these patient's susceptibility to recurrent infections.

4. To examine the functional capacity of leukemic leukocytes.

5. To investigate the mechanisms by which eosinophils kill bacteria.

6. To examine the effect if any that cyclophosphamide has on eosinophil function.

Animal Studies:

1. To investigate the mechanism by which immunologically enhanced guinea pig macrophages acquire an increased capacity to kill chronic intracellular pathogens.

Methods Employed:

In vitro studies of host defense include chemotactic assays (Boyden chamber) and passive migration to study directed and random motility. Phagocytic function is assessed using C^{14} labeled Staphlococcus aureus and thereby quantitating cell associated bacteria with time. Leukocyte bactericidal assays are performed using a pour plate technique. Metabolic functions are assessed by measurements of O_2 consumption (O_2 electrode), hexose monophosphate shunt (C^{14}-1 glucose oxidation) and Krebs cycle (C^{14}-6 glucose oxidation) activity, hydrogen peroxide formation (C^{14}-formate oxidation) and reduction of nitro-blue tetrazolium dyes. Iodination of intracellular protein with phagocytosis is determined by incubating leukocytes with I^{125} and counting radioactivity in TCA precipitates of whole cells.

Human monocytes are separated from whole blood by Ficoll-Hypaque gradient centrifugation, cultured on Petrie dishes or in flasks and their bactericidal and metabolic properties evaluated after 3 to 4 days of incubation and conversion to macrophages.

Guinea pig peritoneal exudate macrophage-lymphocyte mixtures are incubated in the presence and absence of an antigen to which the animal has been previously sensitized. Those macrophage lymphocyte mixtures incubated in the presence of antigen acquire enhanced bactericidal properties and the mechanisms by which this take place can be investigated.

After dextran sedimentation, human blood eosinophils are purified using a hypaque gradient centrifugation technique and the metabolic, and bactericidal properties assessed.

Major Findings:

1. Neutrophils of patients with paroxysmal nocturnal hemoglobinuria were found to have defective phagocytic uptake of radiolabeled staph. aureus and thereby an inability to kill this organism normally.

2. The neutrophils of a patient with a successful bone marrow transplant but subsequent susceptibility to recurring bacterial infections was shown to have defective phagocytic capabilities and diminished bactericidal function. Post-phagocytic metabolic parameters appear to have remained intact.

3. That eosinophils are hypermetabolic at rest and less effective phagocytes as compared with neutrophils was confirmed. The high levels of resting peroxidase activity was shown not to contribute but rather to detract from the eosinophils bactericidal capability. This is in marked contrast to the neutrophil and supports the contention that non-peroxidase related mechanisms can play a significant role in killing intracellular bacteria.

4. Preliminary data suggests that cyclophosphamide treatment of patients with hypereosinophilic syndromes is symptomatically beneficial. A laboratory correlate of efficacy may be a decreasing in the eosinophil's resting hypermetabolic activity.

5. Studies of guinea pig peritoneal macrophages indicate that immunologic enhancement of their bactericidal function against facultative intracellular pathogens is not mediated by the classical H_2O_2 producing pathways of the neutrophil and monocytes. Preliminary studies suggest that macrophages from patients with chronic granulomatous disease kill organisms as well as normal macrophages and support this contention.

Significant to Biomedical Research and the Program of the Institute:

These studies provide insight into the mechanisms which govern normal phagocytic and digestive activity of leukocytes and how they may be altered in various disease states. Such an understanding may conceivably lead to therapeutic correction of abnormalities and enhancement of normal activities when indicated.

Proposed Course:

1. To continue investigations into the mechanisms by which cells phagocytize and kill bacteria. Cells of those patients with abnormal phagocytosis provide tools for investigating these pathways.

2. To study the adequacy of the serum from patients with systemic lupus erythematosis to support opsonization and phagocytosis of bacteria.

3. To continue studying patients with unusual or recurrent infections looking to understand why they may be abnormal.

4. To study the effects of alkylating agents and antimetabolites on leukocyte function.

5. To study the serum and leukocytes from uremic rabbits to look for the abnormality (ies) that may explain the enhanced susceptibility to infections in uremia.

6. To continue studying macrophages to better elucidate the pathways by which they kill bacteria.

7. To expand studies on leukemic leukocytes looking for changes in metabolic function.

Honors and Awards: NONE

Publications: NONE

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LABORATORY OF INFECTIOUS DISEASES

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SUMMARY STATEMENT - Annual Report of the Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases
July 1, 1971 through June 30, 1972

The main interest of LID continues to be the natural history of acute infectious diseases of man and the development of methods for the control or prevention of such illnesses. During the past year, significant progress was made in both of these areas of research.

Hepatitis. Hepatitis continues to be one of the major unsolved infectious disease problems of man. Changing cultural conditions (the rise of the drug culture) have resulted in an increase in the relative importance of the more serious type of disease caused by the virus of hepatitis B. In addition, the use of recently developed, highly sensitive serologic techniques for the detection of antibody indicates that hepatitis B virus infection is more prevalent in the general population than was previously suspected.

We have focused our efforts primarily on the epidemiology of hepatitis B virus infection and on attempts to propagate the virus in the laboratory under experimental conditions. The development of sensitive, specific radioimmunoassay techniques for the detection of HB Ag (an antigen associated with hepatitis B virus infection) and anti-HB Ag (its antibody) described in last year's report, made it possible to attack the problem of hepatitis B virus along these two broad fronts.

Antibody to HB Ag (anti-HB Ag), as measured by radioimmunoassay, was found infrequently in infants and children but was detected with increasing frequency in young and middle aged adults; almost 40% of middle aged adults in a low socioeconomic group population had such antibody. These findings suggested that there was a high sub-clinical attack rate of hepatitis B virus infection in the general population. It seemed unlikely that such a high attack rate could be explained solely by parenteral spread of the virus. Thus, it is probable that the virus commonly spreads by some other means. To further characterize the pattern of infection in various populations, seroepidemiologic studies were performed in a low socioeconomic group (Harlem, New York), a middle socioeconomic group (a Staten Island working class population) and a high socioeconomic group (Park Avenue, New York). In each of the three populations, the pattern of anti-HB Ag antibody acquisition (increasing frequency of antibody with increasing age) was found to be similar but the frequency of antibody in a given age group was inversely related to the socioeconomic status of the population. Thus, infection with the agent of hepatitis B was most prevalent in the low socioeconomic group. The pattern of infection, however, appeared to be different from that of certain viruses spread by the fecal-oral route, such as poliovirus. Infection of infants and children with hepatitis B virus was rare in the open populations sampled. Failure of infants and young children to be infected with hepatitis B virus is probably not related to lack of susceptibility, since children in closed populations, such as homes for the mentally defective, commonly have a high rate of infection.

Sera from over 1500 old world sub-human primates representing the major taxonomic classifications of these animals, were studied for anti-HB Ag. Antibody was found in a variable proportion of sera and in general, there was a direct

relationship between the frequency of anti-HB Ag and the evolutionary status of the species tested. Thus, approximately 50% of chimpanzees tested had evidence of prior infection with the hepatitis B virus whereas less than 5% of macaques had such antibody. Similar studies on new world sub-human primates are currently in progress; preliminary results suggest that these animals are also susceptible to infection with the agent of hepatitis B.

The most notable success of the past year in our hepatitis program was the serial transmission of hepatitis B virus infection in the rhesus monkey. In collaboration with Dr. William London (NINDS) sub-human primates of several species (rhesus monkey, African green monkey, Cebus monkey, Patas monkey, Wooly monkey and Marmoset) were inoculated with HB Ag-positive plasma in an attempt to induce experimental infection. Success was achieved only in the rhesus monkey. Thus far the virus has gone through 5 serial passages in the rhesus monkey with a dilution of $10^{-15.5}$ of the original inoculum. This represents unequivocal evidence for experimental infection and indicates clearly that we have achieved the first bona fide transmission of hepatitis B virus in a laboratory animal. Infection of the rhesus monkey was confirmed by the pattern of appearance of antigen and the subsequent development of antibody. Transient HB Ag antigenemia occurred after an incubation period of 8 to 13 weeks and was followed by the development of anti-HB Ag. This pattern resembled that seen following post-transfusion hepatitis B infection in man. The infected rhesus monkey failed to develop clinical or sub-clinical hepatitis. Nevertheless, the rhesus monkey should prove to be exceedingly valuable for the characterization of the virus of hepatitis B and for development of procedures for the immunoprophylaxis of disease caused by this virus (Purcell, Feinstone).

Acute Non-Bacterial Gastroenteritis. As described last year, we were successful in transmitting acute non-bacterial gastroenteritis, a 24-48 hour self-limited disease, in volunteers. A filterable agent derived from an outbreak of disease in Norwalk, Ohio induced a spectrum of effects in volunteers ranging from predominantly diarrheal disease to an illness in which only vomiting occurred. Prior experimental illness induced resistance to rechallenge with the same inoculum which lasted for at least 4-16 weeks. Studies in which different filtrates were administered to volunteers indicated that the Norwalk agent was less than 66 nm in diameter and likely less than 36 nm. The agent appeared to lack a lipid coat and was resistant to acid pH. In addition, the Norwalk agent retained infectivity after being heated at 60°C for 30 minutes. This type of heat treatment is known to inactivate the RNA containing picornaviruses but has little effect on DNA containing parvoviruses. Thus, the biophysical properties of the Norwalk agent most closely resembled those of the parvovirus group among known animal viruses. Recently we induced disease in volunteers by the administration of stool filtrates from 5 separate outbreaks in addition to the Norwalk outbreak.

Ill volunteers manifested malabsorption of xylose and fat during the acute illness but these abnormalities cleared within 1 week after recovery. Two volunteers who remained asymptomatic had no evidence of malabsorption. In four ill volunteers, intestinal biopsies demonstrated transient deficiency of 3 intestinal enzymes: sugrase, lactase, and alkaline phosphatase. Intestinal enzymes in the well volunteers remained within normal limits. Light and electron microscopic examination of biopsy material revealed definite pathologic

changes during the acute phase of illness, including blunting of the villi, denudation of the brush border, vacuolization of the cytoplasm, and intercellular deposits of proteinaccous electron dense material. However, viral particles were not seen. Two weeks after illness the morphology of the small intestine had returned to normal. Efforts to visualize the Norwalk agent in intestinal biopsy specimens by immunofluorescence (using convalescent sera from individuals whose illness was naturally acquired or experimentally induced) were unsuccessful.

In an attempt to demonstrate in vitro growth of the Norwalk agent, an inoculum which was infectious in volunteers was passaged 5 times in organ culture of fetal human intestine with an estimated final dilution of 10^{-13}. Oral administration of fluids harvested from the fifth passage produced disease in 1 of 5 volunteers. It appears that the Norwalk agent may have grown in vitro, but further studies are needed to demonstrate this conclusively (Dolin, Wyatt, Buscho).

Mycoplasma pneumoniae. As indicated last year new highly sensitive serologic assay systems were needed in order to explore a number of intriguing but unexplained aspects of the epidemiology of M. pneumoniae infection and disease in man. Efforts in this direction led to the development of a radioimmunoprecipitation (RIP) assay for antibody and an improvement in the sensitivity of the complement mediated antibody killing or mycoplasmacidal (MC) test. Both methods proved to be considerably more sensitive for detection of M. pneumoniae antibody than the techniques which have been standard until now. Investigation of the conditions which affect the mycoplasmacidal action of antibody and complement indicated the importance of a filtered suspension of predominantly single organisms, and an appropriate concentration of cations. The mycoplasmacidal effect of antibody and complement resembled the hemolytic reaction in that variation in concentration of either antibody or complement yielded an S-shaped curve. The antigens involved in the mycoplasmacidal and RIP reactions appeared to be the membrane glycolipids which are also of major importance in the complement fixation and metabolism inhibition reactions.

Several facets of the ecology of M. pneumoniae suggested that disease might be caused by reinfection of individuals who had previous contact with the organism. During the past year we investigated this possibility using the new, highly sensitive assay systems for M. pneumoniae antibody. First we studied a group of 7-12 month old infants. In this group, maternal antibody should have disappeared and indeed we found that most sera were free of antibody. However, in the 13-36 month age group a large proportion of sera were positive by both methods, and the frequency of sera containing antibody increased with increasing age. The difference in frequency of antibody between the 7-12 month old infants and the older children was statistically significant. If the MC and RIP tests measure specific antibody it would appear that a considerable amount of infection occurs early in life when M. pneumoniae disease is rare. The possibility that M. pneumoniae infection might be a sensitization disease was examined further by testing pre-infection sera from individuals who subsequently developed M. pneumoniae pneumonia. Sera collected from Marine recruits when they entered their training at Parris Island were assayed for MC and RIP antibodies. Essentially every man who developed M. pneumoniae pneumonia had RIP antibody before exposure to the organism. Mean MC and RIP antibody titers in pre-exposure

sera of pneumonia patients were lower than those seen in a random sample of the Marine recruit population. One could speculate that lower levels of antibody in men who later developed pneumonia were a reflection of their susceptibility to the organism; however, this is clearly speculation.

Previous attempts to detect antibody in local respiratory tract secretions following M. pneumoniae infection had failed, but this failure may have resulted from an insensitivity of the immunologic methods used. We recently studied nasal secretions from infected volunteers using the MC method and were unable to detect development of complement dependent killing antibody. However, using the RIP test it was possible to detect significant development of local antibody in 9 of 13 infected volunteers. Since mycoplasmacidal antibody was not produced locally it may be that the antibody which we measured was similar to that recently described for M. pulmonis, which appeared to be opsonic and required for the clearing of M. pulmonis by mouse macrophages.

The cell killing reaction produced by M. pneumoniae antiserum was shown to be mediated by antibody specific for the glycolipid hapten of the organism. Small circumscribed circular lesions of 90-100 Å in diameter were seen in negatively stained preparations of M. pneumoniae which had been treated with antibody and human complement for a short period of time. Apparently the 90-100 Å holes in the membrane are the initial lesions and these are followed by the development of more extensive holes and tears. This is in good agreement with observations on the effect of antibody and complement on other biological membranes.

Previously the ts mutants of M. pneumoniae induced by the chemical mutagen NTG were thought to be stable genetically during growth in the hamster. In these experiments revertant organisms were sought in lung suspensions which had been frozen prior to testing. Recently when lung suspensions were tested fresh, i.e. without prior freezing and thawing, we found to our surprise that many of these mutants were not stable. This prompted us to seek other NTG-induced mutants which were stable genetically in vivo. Seven such stable mutants have been recovered and characterized. Two of these new mutants had an in vitro shutoff temperature of 38°C, (i.e., they did not form colonies at 38°C or above) while the other 5 mutants shutoff at 37°C. Five of the mutants were studied in the hamster in some detail. The 37°C shutoff mutants were not recovered from the lungs nor did they induce lesions in the lungs. Only one of these mutants, 640, induced protection against subsequent challenge with wild type organisms. In contrast, the two 38°C shutoff mutants, H9 and H10, grew well in the lungs and persisted in this location for at least 80 days and at a higher titer than the wild type. Significantly, the H9 and H10 mutants were completely stable genetically and did not cause lung lesions despite their pattern of pulmonary growth and persistance. Finally, the H9 and H10 mutants induced impressive resistance to challenge with virulent wild type organisms. Because of these encouraging results we are currently preparing suspensions of the H9 and H10 and 640 mutants for evaluation in volunteers to determine the suitability of these mycoplasmas for immunoprophylaxis of human disease caused by M. pneumoniae (Brunner, Chanock).

Temperature Sensitive Recombinants of Influenza A Virus: Genetic Studies and Evaluation in Man. As described before, we produced conditional lethal, temperature sensitive (ts) mutants of influenza A/1965 (H2N2) virus with the

aim of evaluating their attenuation for man and suitability for use in a live virus vaccine. Ts mutation offers the possibility of site-specific attenuation for the lower respiratory tract. Mutants which are markedly restricted in growth at 37°C-38°C should probably be limited in replication in the lower respiratory tract, the major site of significant pathology, which has a temperature of 37°C. However, growth of the mutants should occur with reasonable efficiency in the cooler passages of the upper respiratory tract, which have a temperature of 32°C-34°C.

Previously 2 ts mutants of influenza A/1965 (H2N2) were produced by growing virus in the presence of 5-fluorouracil (5-FU). One mutant, ts-1, failed to produce plaques at 37°C or above, while the other mutants, ts-2, had an *in vitro* shutoff temperature of 39°C. In contrast wild type virus produced plaques in monolayer cultures with high efficiency at 40°C. Both mutants were genetically stable *in vitro* and both were restricted in their growth in the hamster's lower respiratory tract; ts-1 was more restricted *in vivo* than ts-2. Although both mutants grew poorly in the lungs they induced significant resistance in hamsters to subsequent challenge with wild type virus. Finally, the mutants were found to possess distinct genetic lesions.

Encouraged by the behavior of the ts-1 and ts-2 mutants in the laboratory we sought to evaluate their level of attenuation in man. Efforts to study this property of the mutants were frustrated by our failure to find volunteers who lacked serum antibody for these viruses. This was not surprising since the influenza A2 viruses had been epidemic on a number of occasions during the 1957-1968 interval. In order to approach the question of the relationship of virulence and ts mutation we produced recombinant viruses with the surface hemagglutinin (H3) of Hong Kong influenza virus and a ts lesion (or lesions) of the influenza A/1965 (H2N2) ts-1 or ts-2 mutant. This was done since a significant proportion of the adult population in our locality lacked serum neutralizing antibody for the H3 antibody when our studies were performed.

Recombinants derived from the ts-2 mutant had the same temperature sensitivity (i.e., shutoff temperature of 39°C) as the parent mutant. Further these recombinants did not complement each other in pair-wise infection suggesting that they shared a common ts lesion acquired from the ts-2 mutant. The influenza A (H3N2) ts-2[C] recombinant was evaluated in volunteers.

Recombinants derived from the ts-1 mutant exhibited 3 different patterns of temperature sensitivity. Like the parent ts-1 mutant, one class of recombinants had a shutoff temperature of 37°C, while recombinants belonging to the other two classes had restrictive temperatures of 38°C and 39°C, respectively. This suggested that the ts-1 mutant contained 3 discrete ts lesions and that these were segregated during recombination with the influenza A/Hong Kong/1968 (H3N2) virus. This suspicion was confirmed by complementation analysis which showed that the 37°C, 38°C and 39°C ts-1 recombinants possessed distinct genetic lesions. The influenza A (H3N2) ts-1[A] recombinant (shutoff temperature of 37°C) and the influenza A (H3N2) ts-1[E] recombinant (shutoff temperature of 38°C) were evaluated in volunteers. Before the 3 recombinants were administered to volunteers it was ascertained that these viruses were moderately or markedly restricted in their growth in the hamster's lungs. Furthermore, infection of hamsters with the recombinants induced significant resistance to later challenge with wild type influenza A/Hong Kong/1965 (H3N2) virus.

A mutant to be used for immunoprophylaxis of influenzal disease must strike a fine balance with respect to attenuation on the one hand and capacity to stimulate the hosts' protective mechanisms on the other. Two of the recombinants, (ts-1[A] and ts-2[C]), failed to achieve this desired balance. The ts-1[A] recombinant was too attenuated to infect seronegative volunteers, while the ts-2[C] recombinant appeared to be insufficiently attenuated and was thus not acceptable for purposes of immunoprophylaxis.

Fortunately the 3rd recombinant, ts-1[E], appeared to possess the desired degree of attenuation. The virus was capable of infecting almost every seronegative volunteer given 10^6 $TCID_{50}$ by the nasopharyngeal route. However, this virus was sufficiently defective so that the majority of infected individuals did not develop symptoms. Those symptoms which occurred were mild and were considered acceptable when balanced against the disease to be prevented. Infection with the recombinant was extensive enough, however, to stimulate a moderately high titer of serum and nasal wash neutralizing antibody. In view of this immunologic response it was not surprising that volunteers infected with the ts-1[E] recombinant were completely resistant to influenza disease produced by a virulent wild type virus challenge. The ts-1[E] recombinant possessed 2 other properties desirable in a vaccine strain. The recombinant appeared to be genetically stable during replication in the respiratory tract of man and infection did not spread from infected individuals to susceptible cohorts despite close contact during a time when the former group shed virus.

If recombination is the basis for major antigenic shift of influenza A viruses it is ironic and perhaps appropriate that this mechanism may prove to be of major importance in bringing these viruses under control. In our studies genetic recombination was used to transfer ts lesions from a 1965 strain of virus (H2N2) into the 1968 Hong Kong virus (H3N2). Of course, this method of transferring attenuation to a new influenza virus requires that the ts lesions not affect that portion of the genome which codes for the hemagglutinin, the antigen which stimulates neutralizing antibody. Since we were fortunate in inducing mutations in the non-hemagglutinin portion of the genome, transfer of ts lesions to the Hong Kong virus could be achieved. Transfer of ts lesions from influenza A (H2N2) to the Hong Kong virus (H2N2) occurred with high frequency and was accomplished in a short period of time. The facility with which this manipulation was performed suggests that it should be possible to effect a similar transfer of an appropriate ts lesion at the time the next major antigenic variant emerges. This approach offers the possibility for rapid development of a live vaccine to be used for containment of pandemic disease. Since we now have a ts lesion (ts-1[E]) which specifies the appropriate balance of attenuation and immunogenicity required of a vaccine virus the prospects for future live recombinant virus vaccines are reasonably hopeful (Murphy, Chalhub, Chanock).

Specific and Non-specific Resistance to Influenza Virus Infection. Several significant advances were made in our understanding of the factors involved in protection against influenzal disease. During the past year we obtained the first evidence that antibody to the neuraminidase enzyme provides resistance to human influenzal illness. This finding suggests that efforts should be made to stimulate anti-neuraminidase antibody as well as anti-hemagglutinin antibody for more effective prophylaxis of influenza. It should be emphasized

that the anti-hemagglutinin antibody is more protective and can provide impressive resistance in the absence of anti-neuraminidase antibody. However, for maximal protection it would be desirable to induce both types of antibody (Murphy).

One theoretical advantage of a live vaccine over an inactivated vaccine is the possibility that the former might stimulate an initial non-specific resistance before specific immunological resistance develops. If this occurred, the period of protection afforded by a live vaccine could include the first few days after vaccine administration. It was of interest, therefore, to find that the ts-1[E] influenza recombinant induced the development of detectable interferon in 50% of the experimentally infected vaccinees. This suggests that the ts-1[E] virus might induce non-specific resistance to influenza prior to the development of specific immunologic protection.

Evaluation of a Temperature Sensitive Mutant of RS Virus for Immunoprophylaxis. Special emphasis has been given to the development of a live attenuated strain of RS virus to use in infants because of the increasing evidence that local respiratory tract defenses play a major role in resistance to infection. The temperature sensitive ts-1 mutant of RS virus, induced by the chemical mutagen 5-fluorouridine, was chosen for the initial trials on the basis of its behavior in vitro in tissue culture and in vivo in the experimental hamster and in adult volunteers. The mutant, which has an in vitro shutoff temperature of 37°C, infected adult volunteers without producing disease and induced resistance to subsequent challenge with virulent wild type virus. Isolates obtained from the adult volunteers retained their ts property. Encouraged by these findings the ts-1 mutant was evaluated in children of progressively younger age by Dr. Robert Parrott of Children's Hospital of D.C. The mutant was given, by the nasopharyngeal route, to 32 infants and children. Initially older children were studied, and when it was ascertained that the mutant did not induce any signs or symptoms the studies were extended to include younger children and finally, infants. All but one of the 32 infants and children were infected following administration of the ts-1 mutant. In 24 instances this represented reinfection since the children possessed serum neutralizing antibody for RS virus prior to administration of the mutant. Eight of these 24 children developed mild rhinorrhea but no other signs or symptoms of respiratory tract disease; the other 16 children were asymptomatic. Infection of the 7 seronegative infants induced moderate rhinorrhea but no other signs or symptoms of respiratory illness. In one instance, an infant who had rhinorrhea developed otitis media after excretion of virus had ceased, probably as a result of local lymphoid hyperplasia in the nasopharynx and blockage of the eustachian tube.

On occasion, virus with wild type temperature sensitivity, i.e., capacity to produce plaques in tissue culture at 37°C or higher, was recovered from infants and children infected with the ts-1 mutant. Many of the "revertants" were not identical to wild type virus (which exhibited a high efficiency of plaque formation at 37°C, 38°C and 39°C) in that plaque formation was restricted at 38°C or 39°C. This suggests that an additional suppressor mutation had occurred rather than reversal of the original mutation with reversion to the wild type genotype. Direct titration of nasopharyngeal specimens for virus with ts or wild type phenotype indicated that "revertants" represented less than 0.1% of virus produced in the upper respiratory tract. Thus, revertant virus did not

"take over" and become the dominant member of the viral flora present in the nasopharynx of vaccinees. Finally, the occurrence of this low level of "reversion" was not associated with any signs or symptoms.

In summary, the ts-1 mutant was completely attenuated for adults and children all of whom had prior experience with RS virus. In seronegative infants the mutant induced rhinitis which is probably an acceptable price to pay for protection against serious RS virus disease of the lower respiratory tract. However, the development of otitis media following rhinitis is a source of concern which has prompted us to consider initiating clinical evaluation of other ts mutants of RS virus. Ideally, we are seeking a mutant which will (a) infect without undergoing reversion, (b) induce resistance to wild type virus and (c) cause no rhinitis (or milder rhinitis than that caused by ts-1 mutant) in seronegative infants. Currently, efforts are underway to render the ts-1 mutant more stable genetically and also more attenuated for man. We are attempting to introduce an additional ts lesion into the mutant by exposing it to a potent chemical mutagen, ethylmethane sulfonate (EMS), and we will seek clones of virus which are more stable genetically when stressed by incubation at restrictive temperaure (Hodes, Chanock).

Nasal Secretory Antibody Response to Rhinovirus Vaccination. Last year we showed that intranasal vaccination with an inactivated rhinovirus antigen induced resistance to challenge with live virus which lasted for at least six months following vaccination. The local neutralizing antibody which appeared following vaccination was specific for rhinovirus 13 and such local antibody persisted for at least 330 days following vaccination. In the current study intranasal administration of a small quantity of inactivated vaccine did not stimulate a serum or local secretory antibody response in men who lacked these 2 antibodies. However, this quantity of vaccine did stimulate local nasal antibody in men who possessed a low level of both antibodies prior to vaccination. Men with high levels of both antibodies did not respond to vaccination, probably because the high pre-existing antibody titers either dampened a response or obscured it. These findings are compatible with the view that the local respiratory antibody system possesses some degree of immunologic memory (Buscho, Kapikian).

Acute Respiratory Tract Disease in Adults. 566 adults with upper respiratory tract disease have been studied in depth for evidence of viral infection. When all the resources of the virus laboratory were brought to bear upon the problem it was possible to identify virus infection in 52% of the illnesses. Rhinoviruses were detected most frequently followed by coronaviruses. Two of the rhinovirus isolates appear to represent new serotypes and have been submitted to the Rhinovirus Collaborative Program for confirmation of their distinctness.

The most exciting new finding involved the recovery of a new coronavirus by the use of human tracheal organ culture combined with immune electron microscopy. This new virus was not detected by any of our standard culture or electron microscopy techniques, however, the combined organ culture-immune electron microscopy method detected this virus and permitted us to tentatively designate it as a new coronavirus serotype. This new technique, which combines the use of organ culture and the addition of convalescent serum containing antibody to organ culture harvests in order to aggregate virus particles and facilitate their visualization by electron microscopy, offers great promise for the future detection of heretofore unrecognized agent (Kapikian).

Serial No. NIAID 66A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1972 through June 30, 1972

Project Title: Epidemiologic Studies of Infections and Illnesses, and Their Control in Infants and Children

Previous Serial Number: 66A-B

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID

Contract Support:

Contract Project Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	<1/12	<1/12		
Other:	4/12	1/12		5/12

Project Description:

This was a long term, intensive study of the illness and microbial experience of infants and children at Junior Village, a District of Columbia Welfare Institution. This study began in July of 1955 and the field portion of the study was discontinued on May 21, 1969.

Basic Objectives:

The general objective was to maintain under observation a population group suitable for epidemiologic study of occurrence of infections and disease and, in addition, to study host-parasite-disease relationships as they occur naturally and as they could be altered by vaccines and/or chemo-prophylactic agents. A concerted effort was made to (a) find new microbiologic agents which cause disease; (b) find methods of identification of these agents; (c) define modes of spread of infectious agents; and (d) find methods for prevention and treatment of acute illnesses, particularly respiratory illnesses.

Methods Employed and Patient Material:

The field portion of this study was discontinued on May 21, 1969. The epidemiologic setting was described in detail in previous annual reports. Currently, original throat swab specimens from the study are stored at -70°C for the study of coronaviruses when suitable methods for their propagation become available; in addition, anal swab specimens are stored at -20°C for the study of gastroenteritis agents when suitable methods for their propagation are found.

Major Findings:

Epidemiology of Rhinovirus Infection and Characterization of Rhinoviruses - Most respiratory viruses have been studied extensively at Junior Village. However, one major group which has not been studied extensively has been the rhinoviruses since antisera were not available for many serotypes and as a result typing of strains on a limited basis was generally unsuccessful. However, with the completion of the second phase of the rhinovirus collaborative program and the availability of antisera to all 89 serotypes and the one subtype, efforts will be made to study the epidemiology of the numerous rhinovirus serotypes at Junior Village. Thus far, acid lability tests have been carried out on 344 viral isolates from 222 residents and 122 viruses have been found to be acid labile. These studies will be continued in an attempt to determine the epidemiologic pattern of rhinovirus infection in this population.

An Outbreak of Infectious Lymphocytosis - This outbreak occurred in a four month period when at least 22 cases of acute infectious lymphocytosis were discovered. The leukocyte counts varied from 20,000 to 94,000 per mm. with a relative lymphocytosis on the average of approximately 80%. Routine viral, bacterial, fungal, and parasitic studies were performed on specimens obtained during the outbreak and no definitive etiologic agent was found. Renewed attempts will be made to isolate an etiologic agent by using newer techniques such as organ cultures and experimental cell cultures. The occurrence of EB virus infection in 10 children with infectious lymphocytosis was studied using the immunofluorescent technique. These studies which were reported in a previous annual report revealed that none of 6 children without pre-existing antibody, and none of 4 with such antibody developed four-fold or greater EB virus antibody rises. In addition, paired sera from 9 of the 10 children were tested by the CF technique for evidence of infection with cytomegalovirus (CMV) and herpes simplex virus (HSV); no rises to CMV and one to HSV were detected.

Seroepidemiological Studies of Coronavirus Infections - In last year's annual report we reported the incidence of OC43 and 229E virus infections as determined by serologic (CF) tests of paired sera obtained from children admitted to Junior Village between October 15 and December 15 during the years 1961 through 1968. This period had been selected in an attempt to bracket the "winter" seasons with a serum collected on admission to the cottage and a serum taken several months later since it had been shown that adult coronavirus infections were prevalent during both a "winter" and "winter-early spring" period. Since it was found that 23 (16%) of 148 children developed significant CF antibody

rises to OC43 virus (in contrast, however, only 1 of 149 developed such a 229E virus antibody rise), these seroepidemiologic studies have been extended to include sera obtained during all periods of the year. In addition, in these expanded studies, the sera have been tested not only by the CF technique for 229E OC43 virus antibody, but also by the HI technique for OC43 virus antibody. A preliminary comparison of the sensitivity of the HI and CF techniques for detection of OC43 and OC43 virus-related infection has been made: 12 antibody rises were detected by the CF and/or HI techniques--5 of these were detected by the HI technique only and 3 by the CF technique only indicating that both are essential for maximum detection of infection. In addition, study of serially collected sera tested by both techniques indicated that OC43 virus HI antibody persists for longer periods than CF antibody. The relative sensitivity and specificity of these serologic techniques for detection of OC43 virus infection must await the development of sensitive OC43 virus isolation techniques. These seroepidemiologic studies will be continued and further analysis made in an attempt to determine the importance of coronaviruses in this population. Selected throat swab specimens will also be tested in HEI MA177 cell cultures in an attempt to recover coronaviruses from those periods shown by serologic tests to have had a high incidence of coronavirus infections.

Significance to Biomedical Research and the Program of the Institute:

This project represents a systemic approach to determining the various etiologies of acute febrile illnesses. Since carefully gathered epidemiological data and clinical specimens are available, this project also offers an unusual opportunity for the study of the epidemiologic patterns of infections associated with newly discovered agents.

Proposed Course of Project:

The field portion of this study was discontinued on May 21, 1969, when it became apparent that vaccine studies could not be carried out in this population. Studies relating to this project will be continued as long as profitable.

Honors and Awards: None

Publications: None

Serial No. NIAID 66G
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Possible Relationship of Viruses with Systemic Lupus Erythematosis

Previous Serial Number: Same

Principal Investigator: Dr. Neil R. Blacklow

Cooperating Units: Dr. Theodore Pincus, Laboratory of Pathology, NCI
Dr. Phillip Grimley, Laboratory of Pathology, NCI
Dr. Alfred Steinberg, Arthritis and Rheumatism Branch, NIAMD

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12			27/12
Professional:	15/12			15/12
Other:	12/12			12/12

Project Description:

This project is to be discontinued.

Serial No. NIAID 66H
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Etiology of Acute Infectious Non-bacterial Gastroenteritis

Previous Serial Number: Same

Principal Investigators: Dr. Raphael Dolin
Dr. Robert F. Buscho
Dr. Richard G. Wyatt
Dr. Robert M. Chanock

Cooperating Units: Dr. Sol Agus (Laboratory of Digestive and Hereditary Disease)
Dr. Julius A. Kasel (Medical Virology Section, LCI)
Dr. Hubert DuPont and Dr. Richard Hornick (Univ. of Md.)
Dr. Neil R. Blacklow (Boston Univ. School Med.)

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12			27/12
Professional:	15/12			15/12
Other:	12/12			12/12

Project Description

Objectives: (1) To search for viruses which may play an etiologic role in the syndrome of acute infectious non-bacterial gastroenteritis (AINBG); (2) to characterize such new viral agents for their biologic, immunologic, and epidemiologic properties; (3) to cultivate the agents of AINBG in tissue culture, organ culture, or laboratory animals; (4) to experimentally reproduce the syndrome in adult volunteers under controlled conditions, and to study the clinical course of the disease, including the histopathologic, physiologic, and immunologic responses of the host.

Methods Employed:

A. Source of Infectious Material - Stools and paired serum specimens were collected from a variety of naturally occurring outbreaks of AINBG. Particular care was taken to obtain specimens from secondary and tertiary contact cases. Specimens were obtained from (a) outbreaks at Norwalk, Ohio; Denver, Colorado; Tampa, Florida; New Britain, Connecticut; and Morgantown, W. Virginia (Courtesy of Drs. M. Hatch, F. Judson, M. Loewenstein [NCDC]); (b) outbreaks among families of NIH personnel; (c) two separate outbreaks at Henryton State Hospital, Henryton, Maryland and (d) an isolated outbreak in a family in Honolulu, Hawaii (Courtesy of Dr. A. Deane).

B. Processing of Fecal Samples - Stools were examined for the presence of known viral, bacterial, fungal, and parasitic agents, and for the presence of enterotoxin in rabbit ileal loop assay (courtesy of Dr. S. Formal, Walter Reed Army Hospital). 2% stool suspensions were prepared in veal infusion broth with 0.5% albumin, clarified by low-speed centrifugation and filtered through 1200 nm, 800 nm or 450 nm pore sized membranes to exclude bacteria. The filtrates were subjected to rigorous safety tests in tissue culture and laboratory animals (both newborn and adult) to exclude adventitious agents and were examined in the electron microscope. These filtrates which were used as inoculae in volunteer studies were also examined in a variety of experimental systems in an attempt to recover an etiologic agent or define its biophysical properties. In 1970-1971 we transmitted the syndrome through three serial passages by the administration of a stool filtrate from the Norwalk outbreak.

C. Volunteer Studies - During the past year we attempted to define certain biological and biophysical properties of the agent, as well as certain pathophysiological features of the disease using man as the experimental host. To date, 206 volunteers have been studied at the Maryland House of Correction, Jessup, Maryland, and at the NIH Clinical Center. Experimental disease has been entirely self-limited, uncomplicated, and has resolved spontaneously. We have, therefore, felt justified in continuing volunteer studies with the Norwalk inoculae, as well as material from other outbreaks.

D. Intestinal Organ Cultures - Because of the evidence that certain respiratory viruses can be cultivated only in human tracheal organ cultures we attempted to employ an analogous system, human fetal intestinal organ cultures, in the search for viral agents from gastrointestinal disease. These cultures were prepared from fetuses obtained from surgical therapeutic abortions, performed for psychiatric indications, at several regional hospitals. The fetal organ cultures were obtained on a regular basis and could be maintained in sufficient quantity for screening studies and quantitative assays. Employing a variety of known agents, the growth patterns of viruses in these cultures have been studied. Techniques for the detection of viruses in organ culture were developed, employing histologic examination (both light and electron microscopy), immunofluorescence, and enzyme production.

E. Other Culture Systems Employed - Specimens from individuals with naturally occurring or experimentally induced disease as well as organ culture harvests, were inoculated into a wide variety of primary and continuous monolayer tissue cultures. These cultures were observed for cytopathic effects, hemadsorption, viral interference, and evidence of fluorescent antibody stainable antigen. Tissue culture harvests were serially passaged and inoculated cultures were examined at each passage level as above.

Major Findings:

A. Further Characterization of the Norwalk Agent - Prior experimental illness induced resistance to rechallenge with the same inoculum for a period of at

least 4-16 weeks. Volunteer studies involving administration of stool material filtered through gradocol membranes of different average pore size indicated that the Norwalk agent was less than 66 nm in diameter and likely less than 36 nm. The agent appeared to lack a lipid coat and was resistant to acid pH. In addition, the Norwalk agent retained infectivity after being heated at 60°C for 30 minutes; 4 of 17 men who received heated Norwalk agent became ill. This type of heat treatment has been shown to inactivate the RNA containing picornaviruses but has little effect on DNA containing parvoviruses. Thus, the biophysical properties of the Norwalk agent most closely resemble those of the parvovirus group among known animal viruses.

B. Transmission of Acute Gastroenteritis from Other Outbreaks - Recently we induced disease in volunteers by the administration of stool filtrates from 5 separate outbreaks in addition to the Norwalk outbreak. Disease was produced, though with a low frequency, by filtrates from outbreaks in Denver (one of 3 men became ill), Bethesda (1 of 3), and Henryton, Maryland (1 of 3). These agents have been transmitted through an additional passage in volunteers with a similar low frequency of disease. A filtrate from another familial outbreak in Bethesda produced disease in 2 of 4 men, while that from a familial outbreak in Hawaii produced disease in 3 of 4 men. Further passage of these agents in volunteers are currently underway. Clinical features of disease from each outbreak were generally similar, but there was some variation in the incubation period and duration of disease, as well as varying prominence of specific clinical manifestations. In all cases, experimentally induced disease was self-limited and uncomplicated.

C. Clinical Studies - In previous studies, we demonstrated transient malabsorption during experimentally induced gastroenteritis. These findings were confirmed in four ill volunteers, who manifested a transient malabsorption of xylose and fat during the acute illness. These abnormalities cleared within 1 week after recovery. Two volunteers who remained asymptomatic had no evidence of malabsorption. Peroral small intestinal biopsies with the Rubin tubes were performed prior to, during, and two weeks after the acute illness in 6 volunteers at the Clinical Center of the NIH. In four ill volunteers, the biopsies demonstrated transient deficiency of 3 intestinal enzymes, sugrase, lactase, and alkaline phosphatase. Intestinal enzymes in the well volunteers remained within normal limits. Light and electron microscopic examination of biopsy material revealed definite pathologic changes during the acute phase of illness; these changes included blunting of the villi, denudation of the brush border, vacuolization of the cytoplasm, and intercellular deposits of proteinaccous electron dense material. Definite viral particles were not seen. Two weeks after illness the morphology of the small intestine had returned to normal. Frozen sections from intestinal biopsies of two ill volunteers were examined by the indirect immunofluorescence technique, employing convalescent sera from individuals whose illness was naturally acquired or experimentally induced, as well as serum from guinea pigs immunized with concentrated infectious stool. Fluorescent staining was not observed.

D. Studies with Intestinal Organ Cultures - In an attempt to demonstrate in vitro growth of the Norwalk agent, an infectious inoculum was passaged 5 times in organ culture with an estimated final dilution of 10^{-13}. Fluids harvested from this fifth passage produced disease in 1 of 5 volunteers when administered orally. In a previous study, organ culture passed Norwalk agent produced disease in 1 of 5 men, while in another study, disease was not produced in 4 men. Thus, it appears that the Norwalk agent may have grown in vitro, but further studies are needed to demonstrate this conclusively.

The histologic changes induced in organ cultures infected with a series of known viruses were studied. Certain viruses such as Herpes simplex (HSV) and echovirus clearly destroy the cytoartecture of the explants, while others such as adenovirus and AAV grow to high titer without obvious morphologic alteration. We investigated the growth patterns of these viruses employing indirect immunofluorescent techniques with frozen sections of the explants. Adenovirus and AAV FA antigens appeared to be restricted to the nuclei of epithelial cells. Echovirus antigen was seen primarily in the cytoplasm of the epithelial cells, but also in the submucosa. HSV antigen was seen in the nucleus and cytoplasm of cells throughout the explants. Similar immunofluorescent studies in organ cultures, employing infectious AINBG stool suspensions and convalescent sera, failed to reveal the presence of an agent.

Electron microscopy of organcultures infected with the known viruses confirmed light microscopic findings. Intranuclear crystals of adenovirus were seen in epithelial cells, and intracellular arrays of uncoated herpes virus particles as well as extracellular mature virions were noted. Picornaviruses, probably because of their smaller size, were detected less frequently, but intracellular crystals of poliovirus were seen. Organ cultures inoculated with the Norwalk agent, as well as other infectious inocula, showed no distinct histologic changes evident either by light or electron microscopy. Viral particles were not seen within the explants or in the surrounding fluid.

Because an alteration of intestinal enzyme levels was found in volunteers ill with AINBG, the production of enzymes by intestinal organ cultures and the effect of viral infection on these enzymes were studied. Sucrase levels were slightly depressed in adenovirus infected cultures and markedly depressed during HSV infection. Threhalase levels, which rise during incubation in uninfected organ explants, were unaltered during adenovirus infection; in contrast the level of this enzyme decreased during HSV infection. Alkaline phosphatase levels similarly rose in uninfected cultures, but were depressed by both adenovirus and HSV. Norwalk agent did not appear to have an effect on these enzyme levels in intestinal organ cultures.

Currently, studies are underway employing pulse labelling with radioactive nucleotides (thymidine and uridine), followed by rate zonal and isopycinic centrifugation in an attempt to demonstrate presence of viral nucleic acids associated with particles which have the biophysical properties of a virus.

E. Other Studies Involving Conventional Tissue Culture Systems and Biophysical Techniques - Further passages in conventional tissue culture systems using standard detection methods failed to reveal the presence of an agent. Employing the modified hemadsorption inhibition techniques of Carver in WI38, HEK, AGMK cells, no evidence of viral growth was detected.

Stool filtrates were concentrated by aquacide dissication and isopyncnic centrifugation in a short, steep cesium chloride gradient. These preparations were employed in immunodiffusion and complement fixation tests with acute and convalescent sera, as well as in immunization of laboratory animals. To date, the results of these efforts have been negative.

Significance to Biomedical Research and the Program of the Institute:

Acute infectious non-bacterial gastroenteritis is a major widespread infectious disease of uncertain etiology that affects a significant proportion of our population. Identification of an etiologic agent or agents represents an initial step toward the ultimate goals of prevention and therapy. The volunteer studies should continue to provide helpful information on the etiology, epidemiology, and pathogenesis of this disease. Newly developed detection techniques employing intestinal organ cultures offer promise for the study of these agents in the laboratory.

Proposed Course of Project:

Intensive study of etiologic agents from outbreaks of AINBG will continue. The main emphasis will be on the infectious material now available from volunteer experiments. These specimens will be subjected to various biophysical separation procedures in an attempt to recover the virus. Selected new outbreaks of AINBG will be similarly investigated. New agents will be studied with respect to their biologic, immunologic and clinical characteristics, employing the entire panoply of tissue culture and organ culture techniques. Homologous and heterologous immunity will be studied in volunteers by the method of cross-challenge.

Honors and Awards:

Participant in Clinical Conference, NIH Clinical Center, October 17, 1971.

Publications:

Dolin, R., Blacklow,,N.R., DuPont, H., Buscho, R.F., Kasel, J.A., Hornick, R.B., and Chanock, R.M.: Biologic Properties of Norwalk Agent and Acute Infectious Non-bacterial Gastroenteritis. Proc. Soc. Exp. Biol. Med. 140: 578-583, 1972.

Blacklow, N.R. (Moderator). Discussants-Dolin, R., Fedson, D.S., DuPont, H., Northrup, R.S., Hornick, R.B., and Chanock, R.M.: Acute Infectious Non-Bacterial Gastroenteritis: Etiology and Pathogenesis. Ann. Intern. Med., In press, 1972.

Serial No. NIAID 66I
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Natural History and Defective Nature of Infection with Adeno-Associated Viruses (AAV)

Previous Serial Number: Same

Principal Investigator: Dr. Raphael Dolin

Other Investigators: Dr. James Rose (Laboratory of Biology of Viruses)
Dr. Alan Rabson (Laboratory of Pathology)
Dr. M. David Hoggan (Laboratory of Viral Diseases)
Dr. Neil R. Blacklow (Boston Univ. School of Med.)

Contract Support:

Contract Project Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	18/12			18/12
Professional	6/12			6/12
Other:	12/12			12/12

Project Description:

Objectives: (1) To study the biological and immunological properties of AAV; (2) to investigate the relationship between AAV and its helper viruses, adenoviruses and herpesviruses.

Methods Employed:

The AAV are small defective DNA viruses that are able to replicate completely in cell cultures only when coinfected with helper adenovirus. Partial replication (production of fluorescent stainable antigen) occurs when cultures are infected with a variety of herpesviruses. AAV infection in man appears to be widespread, since 50 to 70% of young adults possess neutralizing antibodies against AAV types 1, 2, and 3. However, infection with AAV has not been etiologically associated with human disease.

A. Assay Methods - Human embryonic kidney cells, Hep-2 cells or K-B cells are infected with AAV and helper virus. Since AAV does not produce cytopathic effects, the viruses are assayed primarily by immunologic techniques. AAV specific antigen are detected by complement fixation (CF) and immunofluorescence (FA) tests. The presence of antigen is employed as an end point in infectivity and neutralization titrations.

B. Biophysical Techniques - Purified AAV, free of detectable adenovirus, is prepared by isopycnic gradient centrifugation in Cesium chloride. This preparation serves as the antigen in the CF tests and in the hyperimmunization of guinea pigs to produce monospecific immune serum.

Major Findings:

A. Infectivity of AAV Particles (strand specific) - AAV particles are known to contain single stranded DNA. Rose and Koczot reported two types of complementary strands. One contains 26.5% thymidine (heavy strands) and the other has 21.7% thymidine (light strand). These strand species were substituted with 5'BUDR, and then purified by multiple isopyicnic centrifugation in cesium chloride and rate zonal sedimentations in sucrose. The purified light and heavy particle preparations were found to have the same infectivity in human embryonic kidney cells (HEK) using adenovirus as a helper. Both CF and FA were employed and points in infectivity assays ($TCID_{50}$). However, each population of particles was estimated to be only 80-90% pure (i.e. containing only the light or heavy particles), and further purification of particles and/or a more sensitive assay system must be developed before concluding that both types of particles are equally infectious.

B. Mechanism of Adenovirus Helper Function for AAV - In collaboration with Dr. J. Rose, the kinetics of AAV and adenovirus nucleic acid synthesis were studied. AAV viral specific DNA and RNA were obtained by pulse labeling cultures of adenovirus and AAV in K-B cells with tridiated thymidine and uridine. The nucleic acids were extracted and hybridized with homologous purified viral DNA and RNA. When the viruses infect simultaneously, the appearance of AAV DNA and RNA is temporally associated with the appearance of the corresponding adenovirus DNA and RNA. If cells are preinfected with adenovirus, both AAV DNA and RNA appear early, but with a greatly decreased interval between AAV DNA and AAV RNA production. This suggests that adenovirus provides at least two specific helper functions (one for AAV DNA and one for AAV RNA).

The helper function of adenovirus was further studied by employing ts mutants of adenovirus 31, obtained through the courtesy of Drs. Shimojo and Rose. In an earlier study Ito reported the potentiation of AAV by an adenovirus ts mutant which did not produce DNA at the restrictive temperature. This same mutant in our hands produced (leaked) $10^{0.5}$ to $10^{1.5}$ infectious doses of adenovirus at the restrictive temperature as assayed by plaque titration on HEK. However, no potentiation of AAV was noted when infectious adenovirus was not produced.

Further studies are planned employing more stable and non-leaky mutants of adenovirus.

C. Enhancement of AAV by a Simian Herpes Virus (H. saimiri) - Human herpesviruses have been shown to provide a partial helper function for AAV. In collaboration with Dr. A. Rabson on H. saimiri strain, passed through Marmosets, was similarly studied. H. saimiri is oncogenic for squirrel monkey as well as for Marmosets. The virus is grown in suspension of Marmoset lymphocytes, and can be detected by a lytic cycle in cultivated vero cells. Marmoset lymphocytes infected simultaneously with H. saimiri and AAV-2 produced intranuclear FA stainable AAV antigen (0.1%-1% of cells), but no infectious AAV in the extracellular fluid. Preliminary results with serial sections of cryoform embedded lymphocyte pellets, stained with H. saimiri antiserum (courtesy of Dr. F. Dienhardt), and AAV antiserum suggest that only cells which contain the H. saimiri antigen potentiate AAV. By assaying infections center on vero cells, AAV appears to have a slight but definite inhibition on the number of cells containing H. saimiri.

Significance to Biomedical Research and the Program of the Institute:

Though AAV viruses commonly infect man, their significance to human disease remains unknown. Because of the pathogenic and oncogenic potential of their adenovirus and herpesvirus helper viruses the relationship of AAV to these viruses may be of medical importance. They also provide a unique opportunity to study the biology of viral interactions, and they represent a potentially powerful tool with which to investigate basic molecular mechanisms of defective as well as helper virus functions.

Proposed Course of Project:

The potential role of AAV in human disease, acting by itself or in concert with its helper virus, will continue to be investigated. The mechanisms of the helper functions of adenoviruses and herpesviruses will also continue to be studied.

Honors and Awards: None

Publications: None

Serial No. NIAID 69A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Non-bacterial Respiratory Disease
A. Children

Previous Serial Number: Same

Principal Investigator: Dr. Robert M. Chanock

Other Investigators: Dr. David Hodes
Dr. Albert Z. Kapikian

Cooperating Units: Dr. R.H. Parrott, Children's Hospital Research Foundation, Washington, D.C.

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Project. Dir.	Annual Funding Level
PH 43-68-1309 Part B	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589
NIH NIAID 71-2091	Dr. Chanock	Children's Hosp. of D.C.	Dr. Parrott	$ 141,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	Total
Total:	24/12			24/12
Professional:	6/12			6/12
Other:	18/12			18/12

Project Description

Objectives: (1) To search for new agents responsible for respiratory disease in infants and children; (2) to delineate the epidemiology and pathogenesis of viruses which have been recently associated with respiratory disease; (3) to define the major determinants of host resistance to infection and illness; (4) to continue the surveillance of viruses previously established as playing a role in respiratory disease, i.e., the epidemiology and contribution to overall respiratory disease experience during infancy and childhood; (5) to evaluate experimental vaccines for safety, antigenicity, and effectiveness against naturally occurring respiratory tract disease.

Methods Employed:

Infants and children with respiratory disease and suitable control subjects without such disease are studied at the Children's Hospital, District of Columbia. The main emphasis is on the use of various tissue culture systems and artificial media for the recovery of viruses and mycoplasmas. All specimens from the patients are inoculated directly into tissue cultures and onto mycoplasma media without prior freezing and thawing, if feasible. In addition to specimens for isolation of viruses and mycoplasma, serial nasal washings are taken to study respiratory tract secretory antibody response.

After the various agents have been identified, their contribution to the different respiratory disease syndromes is estimated by comparing the recovery rate from such groups with that observed for healthy children free of respiratory symptoms. In addition, children with severe respiratory disease admitted to the hospital as well as comparable control subjects, are studied serologically for evidence of infection with the known respiratory viruses and mycoplasmas as well as any new agents which might emerge as potentially important pathogens during the course of the study.

Respiratory viruses which are attenuated by exposure to a chemical mutagen and which exhibit no virulence for adult volunteers are administered to children under carefully controlled conditions in the isolation unit of the Children's Hospital Clinical Research Center. As in other studies, parental consent is obtained in each instance. Vaccinees are observed closely for evidence of illness and specimens are taken for the study of virus excretion pattern, serum antibody response and local secretory antibody response.

Major Findings:

(1) Evaluation of a Temperature Sensitive Mutant of RS Virus as a Potential Vaccine Strain - Special emphasis has been given to the development of a live attenuated strain of RS virus for use in infants because of the increasing evidence that local respiratory tract defenses play a major role in resistance to infection. The ts-1 mutant of RS virus, induced by the chemical mutagen 5-fluorouridine, was chosen for the initial trials on the basis of its behavior in vitro in tissue culture and in vivo in the experimental hamster host and in adult volunteers. This mutant did not produce plaques at 37°C and above, unlike wild type virus which produced plaques without restriction at 39°C. In the hamster, infection was limited to the cooler upper respiratory tract (32°-34°C) and virus was not found in the lungs (37°C). The mutant was stable in culture and in hamsters, i.e., there was no evidence of reversion to the wild phenotype. The mutant infected adult volunteers without producing disease and induced resistance to subsequent challenge with virulent wild type virus. Isolates obtained from the adult volunteers retained their ts property.

Encouraged by these findings the ts-1 mutant was evaluated in children of progressively younger age by Dr. Robert Parrott of Children's Hospital of D.C. The mutant was given, by the nasopharyngeal route, to 32 infants and children. Initially older children were studied. When it was ascertained that the mutant did not induce any signs or symptoms the studies were extended to include younger children and finally, infants. All but one of the 32 infants and children was infected following administration of the ts-1 mutant. In 24 instances this represented reinfection since the children possessed serum neutralizing antibody for RS virus prior to administration of the mutant. Eight of these 24 children developed mild rhinorrhea but no other signs or symptoms of respiratory tract disease; the other 16 children were asymptomatic. Infection of the 7 seronegative infants induced moderate rhinorrhea but no other signs or symptoms of respiratory illness. In one instance, an infant who had experienced rhinorrhea developed otitis media after excretion of virus had ceased, probably as a result of local lymphoid hyperplasia in the nasopharynx and blockage of the eustachian tube.

On occasion, virus with wild type temperature sensitivity, i.e., capacity to produce plaques in tissue culture at 37°C or higher, was recovered from infants and children infected with the ts-1 mutant. Many of the "revertants" were not identical to wild type virus (which exhibited a high efficiency of plaque formation at 37°C, 38°C and 39°C) in that plaque formation was restricted at 38°C or 39°C. This suggests that an additional suppressor mutation had occurred rather than reversal of the original mutation with reversion to the wild type genotype. Direct titration of nasopharyngeal specimens for virus with ts or wild type phenotype indicated that "revertants" represented less than 0.1% of virus produced in the upper respiratory tract. Thus, revertant virus did not "take over" and become the dominant member of the viral flora present in the nasopharynx of vaccinees. Finally, the occurrence of this low level of "reversion" was not associated with any signs or symptoms.

In summary, the ts-1 mutant was completely attenuated for adults and children all of whom had prior experience with RS virus. In seronegative infants the mutant induced rhinitis which is probably an acceptable price to pay for protection against serious RS virus disease of the lower respiratory tract. However, the development of otitis media following rhinitis is a source of concern which has prompted us to consider initiating clinical evaluation of other ts mutants of RS virus. Ideally, we are seeking a mutant which will (a) infect without undergoing reversion, (b) induce resistance to wild type virus and (c) cause no rhinitis (or milder rhinitis than that caused by ts-1 mutant) in seronegative infants.

(2) Epidemiology and Pathogenesis of RS Virus Disease - The unusual age distribution of RS virus disease remains unexplained. RS lower respiratory tract illness occurs with highest incidence at 2-3 months of age. It has been suggested by Gardner that this type of RS illness represents a response to the virus by an infant sensitized by prior infection in the first few weeks of life. Detailed epidemiologic analysis of 11 consecutive yearly RS epidemics in the Washington, D.C. area revealed that the temporal pattern of bronchiolitis

and pneumonia in young infants (1-3 months of age) was virtually identical to that of older infants and children hospitalized or seen in the clinic with RS virus disease. If prior infection were a requisite for development of infantile bronchiolitis one would expect to see a lag between peak occurrance of RS infection in children and serious RS obstructive disease in young infants. Since this was not observed it appears that prior encounter with the agent is not required for the development of serious disease in infancy.

The greatest impact of RS virus as a respiratory pathogen occurs in early infancy at a time when a moderately high level of passively acquired neutralizing antibody is present in serum. Thus, infants 1-4 months of age with RS bronchiolitis or pneumonia possess moderate to moderately high titers of RS neutralizing antibody in their acute phase serum. These levels of antibody are comparable to these present in the serum of age matched control infants who are free of respiratory tract disease at the time they are sampled. For the most part the antibody detected in these infants, with or without RS virus illness, appears to be passively acquired from the mother since in each group the highest mean titer is seen in the youngest infants (1 month of age) and there is a progressive decrease in mean titer with increasing age at a rate consistent with decay of passively acquired gamma globulin.

It is of interest that a significant proportion of infants who are 6-7 months of age at the time they are ill with RS bronchiolitis lack detectable neutralizing antibody in their acute phase sera. This finding casts some doubt upon the mechanism previously proposed by us for the pathogenesis of RS virus bronchiolitis--namely immunological injury resulting from a reaction between viral antigens and serum antibody in the lung. Clearly, it is difficult to give serious consideration to this proposed mechanism unless small amounts of serum antibody which cannot be detected by the sensitive plaque assay system are capable of mediating immunologic damage.

Significance to Biomedical Research and the Program of the Institute:

Respiratory disease is the most common ailment of man, particularly during infancy and early childhood when many of the most serious expressions of such illness occur. Detailed knowledge of the agents responsible for respiratory tract disease and an understanding of their natural history are necessary before effective prevention can be achieved.

One of the most pressing needs in respiratory disease control is the prevention of serious RS virus illness during infancy. The present efforts at immunoprophylaxis utilizing the ts-1 mutant of RS virus are promising, however, it is now clear that this mutant has several undesirable properties which probably disqualify it as a vaccine strain for widespread use in infants. Specifically, during infection of young vaccinees the ts-1 mutant occasionally undergoes mutation toward wild type (suppressor mutation) or to wild type (reversion). In addition, the mutant induces moderate rhinorrhea in seronegative vaccinees and in one instance this was associated with the subsequent

development of bacterial otitis media. This information provides us with a helpful frame of reference for evaluation of other ts mutants of RS virus in future studies. In other words, we have now achieved a sharper definition of the properties required for a satisfactory RS vaccine strain. Although we have not developed the definitive RS virus vaccine strain we have moved closer toward our goal and the obstacles which remain to be overcome are clearly in view.

Proposed Course of Project:

It is planned that this study will continue for a period of years since a large segment of respiratory disease in infancy and childhood remains to be explained--approximately 40%. In addition, viruses responsible for respiratory illness vary in different times and in different localities; therefore, surveillance should continue in order to comprehend the larger picture. Efforts to increase our understanding of the pathogenesis of viral and mycoplasmal lower respiratory tract disease of infancy and childhood will continue. Particular attention will be given to elucidating the possible role of sensitization in the pathogenesis of M. pneumoniae pneumonia.

Evaluation of ts mutants of RS virus in children and subsequently in infants will continue. Clinical trials will be performed with ts mutants which appear to be more defective than ts-1. Such mutants are likely to be more stable in vivo than the ts-1 mutant and also less virulent.

Honors and Awards:

Director International Reference Laboratory for Respiratory Viruses other than Influenza, World Health Organization.

Director International Reference Laboratory for Mycoplasmas, World Health Organization.

Member International Subcommittee on Taxonomy of the Mycoplasmatales, International Committee on Nomenclature of Bacteria of the International Association of Microbiological Societies.

Member Commission on Acute Respiratory Diseases, Armed Forces Epidemiology Board, Washington, D.C. Participated in meeting on A.R.D. and Strep/Staph Diseases, November, 1971. Walter Reed Army Medical Center, Washington, D.C.

Associate Member Commission on Influenza, Armed Forces Epidemiology Board, Washington, D.C. Participated in meeting at Walter Reed Army Medical Center, November, 1971.

Served on Editorial Boards of American Journal of Epidemiology, Journal of Immunology, American Journal of Diseases of Children and Journal of Infectious Diseases.

Elected foreign member of the South African Society of Pathologists, 1971

Consultant to World Health Organization Advisory Group on Nomenclature of Influenza Viruses, Geneva, Switzerland, September 30 - October 1, 1971.

Rapporteur World Health Organization, Consultation on Live Influenza Virus Vaccine, Geneva, Switzerland, October 4, 5, 1971.

Participant in 1st International Conference on Immunology, Washington, D.C., August 2 - 6, 1971.

Publications:

Blacklow, N.R., Hoggan, M.D., Sereno, M.S., Brandt, C.D., Kim, H.W., Parrott, R.H., and Chanock, R.M.: A Seroepidemiologic Study of Adenovirus-Associated Virus Infection in Infants and Children. Amer. J. Epidem. 94: 359-366, 1971.

Brandt, C.D., Kim, H.W., Jeffries, B.C., Pyles, G., Christmas, E., Reid, J.L., Chanock, R.M., and Parrott, R.H.: Infections in 18,000 Infants and Children in a Controlled Study of Respiratory Tract Disease. II. Variation in Adenovirus Infections by Year and Season. Amer. J. Epidem. 95: 218-227, 1972.

Chanock, R.M.: Immunological Aspects of Respiratory Virus Infections. 10th International Congress for Microbiology (Colloquium on Respiratory Virus Infections), August 9-15, 1970, Mexico, In press, 1972.

Chanock, R.M., Kapikian, A.Z., Perkins, J.C., Parrott, R.H.: Vaccines for Non-bacterial Respiratory Diseases Other than Influenza. International Conference on the Application of Vaccines Against Viral, Rickettsial, and Bacterial Diseases of Man. Pan American Health Organization, WHO, Washington, D.C., December 14-18, 1970, pp. 101-116.

Kim, H.W., Arrobio, J.O., Pyles, G., Brandt, C.D., Camargo, E., Chanock, R.M. and Parrott, R.H.: Clinical and Immunological Response of Infants and Children to Administration of Low-temperature Adapted Respiratory Syncytial Virus. Pediatrics 48: 745-755, 1971.

Serial No. NIAID 69C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Etiology of Acute Respiratory Diseases in Adults

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr.W Lee Cline, Serology Unit, LID, NIAID
Dr. James MacLowry, Clinical Center, Clinical Pathology
Infections Laboratory Service
Dr. Wilmer Dockstader, Bacteriology Unit
Environmental Services Branch

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	33/12	2/12		35/12
Professional:	6/12	1/12		7/12
Other:	27/12	1/12		28/12

Project Description:

Objectives: (1) To determine the role of known viruses and bacteria as etiologic agents of acute upper respiratory illnesses in adult populations; (2) to find and characterize new agents which may be human respiratory tract pathogens; (3) to investigate the sensitivity of new experimental cell culture systems for the isolation of rhinoviruses and the recently described human coronaviruses.

Methods Employed:

A. National Institutes of Health (NIH) Volunteer Study - The current study began on September 9, 1965 and continues to the present. (An earlier similar study began in 1962 and was terminated in 1964). Employees of NIH with respiratory illnesses volunteer a nasopharyngeal washing, a throat swab, and a blood specimen during the acute phase of a respiratory illness. An additional

nasopharyngeal washing and blood specimen are obtained 3-4 weeks later when the volunteer has recovered from the illness. All of the acute nasopharyngeal washings are routinely inoculated into cell cultures of primary monkey kidney (MK), Hep-2, HDCS WI38, primary human embryonic kidney, human embryonic intestine MA177, and in addition, recently into BS-C-1 cells also., HDCS WI26 cultures and human aorta (AT-39) cell cultures were also used earlier in the study. In addition, the first 50 convalescent nasal washings and subsequently 1 of 4 such washings obtained through October 28, 1968, were tested in cell cultures also. Guinea pig erythrocytes are employed in hemadsorption tests in monkey kidney cell cultures for detection of myxoviruses whereas rat erythrocytes are employed similarly in BS-C-1 cell cultures for detection of OC43 related coronaviruses; rat red blood cells have been found recently to hemadsorb to OC43 virus-infected cell cultures, a finding reported in last year's annual report.

The throat swab is tested for beta (B-) hemolytic streptococci (in our laboratory), and in addition, beginning in March, 1969 for hemophilus influenzae, streptococci and other bacteria, by the Clinical Center Clinical Pathology Infections Laboratory Service (Dr. MacLowry); if B-hemolytic streptococci are isolated they are grouped by the Infections Laboratory Service. Some of the nasal washings have also been tested for staphylococci with subsequent phage typing of recovered organisms (Dr. Dockstader).

In an attempt to find new agents, acute illness specimens which were negative for known viruses in the tissue cultures outlined above, are passaged three times in human embryonic tracheal organ cultures. The third passage harvest is clarified, concentrated, negatively stained with phosphotungstic acid and examined by electron microscopy for the presence of coronavirus-like particles. In addition, the harvest from the third passage is inoculated into the tissue cultures outlined above to determine if a cytopathogenic agent is recoverable. Recently, the additional technique of "immune electron microscopy" has been employed in an attempt to recover new agents, notably coronaviruses, from nasal washings which have failed to yield viral pathogens by tissue culture methods. A description of these studies is presented under Project 72A 'New Techniques for Recovery of Respiratory Tract Viruses."

Major Findings:

From September 9, 1965 to March 31, 1972, a total of 667 volunteers enrolled in the NIH, LID Common Cold Study. From 0 to 35 entered the study each month; some were in the study more than once. At least one viral agent was recovered from 268 (40%) of the 667 study patients; only 2 patients yielded more than one viral agent. Beta hemolytic streptococci were recovered from 24 study patients (2 were group A, 3 group B, 4 Group C, 3 Group F, 7 group K, 2 non-groupable, and 3 not grouped). Two volunteers shed both a B-hemolytic streptococcus (not grouped) and a rhinovirus-like agent, and one shed a B-hemolytic streptococcus (not grouped) and an adenovirus; eight shed both a B-hemolytic streptococcus belonging to groups other than A and a viral agent. Two volunteers shed 2 viruses simultaneously--one both parainfluenza type 3 and an adenovirus, and the other both a rhinovirus and parainfluenza virus type 2.

178 (66%) of the 270 virus isolations were classified as rhinoviruses (171 were acid labile, chloroform stable, and 7 were not tested), whereas 18 (7%) were identified as adenoviruses, 9 (3%) as herpes simplex, 10 (4%) as influenza A2, 7 (3%) influenza B, 6 (2%) parainfluenza type 1, 9 (3%) parainfluenza type 2, 4 parainfluenza type 3, 2 parainfluenza type 4, 5 respiratory syncytial, 19 (7%) coronaviruses (12 "229E-like," one each of OC strains OC37, 38, 43, 44, 48, 692, and 1 untyped presumptive coronavirus tissue culture strain) and 2 unclassified viruses.

As shown above, rhinoviruses were by far the most frequently recovered group of agents having been recovered from 27% of the study patients. However, the epidemiological pattern of rhinovirus infections has been noteworthy as the frequency of such infections has decreased during all 7 "winter seasons" of the study (December, January and February) in comparison to periods of higher prevalence during preceding "fall seasons" (September, October and November). For the 7 years combined, rhinoviruses have been recovered from 90 (37%) of the 243 study patients during the "fall periods", and from only 16 (8%) of 189 during the "winter periods". This difference in rhinovirus isolation rate is statistically significant at the .0001 level. During the "winter periods" other known respiratory virus agents were also recovered infrequently using conventional techniques.

Attempts to recover the etiologic agents of respiratory illnesses occurring during these fallow periods have been a major effort of this laboratory. These efforts have been fruitful with the use of two different culture techniques: human embryonic tracheal organ cultures and human embryonic intestine MA177 cell cultures. Details on the use of such cultures have been presented in previous annual reports and additional recent information on their use is presented in project number 72A. In brief, 10 of the 19 definite or presumed coronaviruses recovered from this study were isolated from nasal washings obtained in December, January, or February, 8 from washings obtained in March, and another in April. Although specimens obtained in the "winter" or "winter-early spring periods" have been tested most intensively, serologic studies from two such periods support the view that coronavirus infections with OC43 and 229E viruses may be important etiologic agents of acute upper respiratory illnesses in adults. In these serologic (CF) studies of paired sera presented in previous annual reports, coronaviruses were the predominant identifiable infecting organisms associated with non-influenzal adult respiratory disease.

Paired sera obtained from patients admitted to the study during the 5 year period from September 1965 through August 1970 were tested for serologic (CF) evidence of infection utilizing numerous respiratory agent antigens. 108 (20%) of 541 patients developed 4 fold or greater CF antibody rises; 11 of the 108 had simultaneous increases in antibody to two agents belonging to different antigenic groups for a total of 119 rises. Of these, 16 were to the para-influenza viruses, 3 to adenovirus, 7 to RS virus, 17 to influenza A virus, 9 to influenza B virus, 28 to coronaviruses OC38 and/or OC43 and/or MHV-A59, 32 to coronavirus 229E, 4 to herpes simplex virus and 3 to M. pneumoniae. It is noteworthy that approximately one-half (60 of 119) of the CF antibody increases were to the coronavirus antigens. Further analysis of the data revealed that

specimens from 566 study patients were tested by both virus isolation and serologic techniques for evidence of infection during the five-year period, September 1965 through August 1970, and evidence of infection by either virus isolation or serologic (CF) techniques or both was found in 296 (52%) of the 566 study patients. In addition, 2 patients had a group A beta hemolytic streptococcal infection. It was of interest that 70 (12%) of the 566 patients had serologic or viral isolation evidence of coronavirus infection--34 (6%) with 229E, 28 (5%) with members of the OC43 group, and 10 (2%) with other definite or presumed coronaviruses.

Seroepidemiologic Studies of Coronavirus Infections in a College Population -

In 1964 we described a pilot study of acute respiratory illnesses of University of Maryland students in which only 2 viruses (parainfluenza types 1 and 4) were recovered from 210 students; in addition, in an intensive serologic survey utilizing 21 CF antigens, serologic evidence of infection could be demonstrated in only 30 (25%) of 122 students from whom paired sera were available. Influenza B virus accounted for over one-half of these infections. The incidence of rhinovirus infections was not ascertained since methods for their study were not known and when such methods became known, the throat swab specimens were no longer available. With the recent discovery of coronaviruses of human origin and their association with respiratory illnesses, we attempted to determine the incidence and prevalence of coronavirus infections in this college population by testing the paired sera still available from the previous study utilizing the CF, HI, and the recently described neutralization-hemadsorption techniques. The CF tests had been performed in 1969 whereas the HI and neutralization-hemadsorption tests were performed this past year.

Paired sera from 110 of the 122 students whose sera were previously studied were available for testing by the CF technique for antibody to OC38, OC43, MHV-A59, and 229E viruses. 19 (17%) of the 110 students developed significant coronavirus CF antibody rises--12 to 229E and 7 to OC38 and/or OC43 and/or MHV-A59 viruses. The latter three strains were grouped together because of their previously described antigenic relationships and the frequent sharing of antibody responses to two or more of this group by individuals with coronavirus infections. Paired sera from 93 of the students were also tested by the HI technique for OC43 virus antibody and no significant antibody increases were found; only 2 of the 7 paired sera demonstrating CF antibody rises were available for the HI antibody studies. It was of interest that 3 of 11 students with 229E virus antibody rises (one student was excluded because of anti-complementary activity in his acute serum) possessed 229E virus CF antibody in acute sera, and 2 of 7 with CF antibody rises to one or more of the OC38 virus group had such antibody to at least one of the antigens to which a rise had occurred. It was also noteworthy that 12 of the 19 students with serologic evidence of coronavirus infection had either an afebrile rhinitis or an upper respiratory illness, 2 an afebrile non-exudative pharyngitis, 2 an afebrile exudative pharyngitis, 2 an influenza-like illness and 1 a pneumonitis.

The prevalence of antibody to these viruses varied and depended upon the technique used for its measurement. 26% of the students with available sera had CF antibody titers of $\geq$1:8 to 229E virus, 19% to OC38 virus, 36% to OC43

virus, and 11% to MHV-A59. (In the CF test an acute serum was tested only if the convalescent serum had a titer of >1:8 in the screening test; in such an instance the acute serum titer was included in the prevalence data tabulation. If the acute serum titered 1:4, it was tabulated at <1:8; if the convalescent serum titered <1:8, this value was included in the prevalence data tabulation.) Prevalence of OC43 virus antibody was also studied by the HI and neutralization-hemadsorption techniques using acute phase sera. By HI technique, 84% of the students had titers of >1:8, whereas by the neutralization-hemadsorption test, all had titers of >1:8; it was noteworthy that 90% of the neutralization antibody titers were >1:32. A comparison of HI and neutralizing antibody titers was also made; identical titers or titers varying only two-fold were found for one-third of the sera whereas in the remainder the neutralizing antibody titers were at least 4 fold greater than those determined by the HI technique. 229E virus neutralizing antibody titers in acute phase sera were also determined and 39% of the students had titers of >1:8; the 229E titers were not nearly as high as those observed for OC43 virus, as only 5% were >1:32. These prevalence data indicate that infection with OC43 or OC43 related agents is very common whereas 229E virus infection occurs less frequently. It appears from this and previously reported studies that OC43 virus infections in adults occur predominantly as reinfections, a pattern similar to that observed with both RS and parainfluenza 3 virus infections in adults. The relative importance of the coronaviruses as etiologic agents of acute respiratory diseases must await the development of suitable sensitive methods for their cultivation.

Significance to Biomedical Research and the Program of the Institute:

These studies are aimed at evaluating the role of known viruses and bacteria as etiologic agents of adult respiratory diseases and at finding new agents which cause these illnesses. It was noteworthy that with the use of both virus isolation and serologic techniques over 50% of the illnesses studied among NIH, LID Common Cold Study volunteers were found to be associated with a viral agent. In addition, the recovery of coronaviruses during certain "winter" and "winter-early spring" periods when non-influenzal respiratory illnesses were prevalent but virus isolation rates paradoxically low was noteworthy. It appears that with the use of new techniques such as organ cultures and experimental cell cultures new agents can be recovered during such previously fallow periods. Elucidation of the etiology of the majority of upper respiratory illnesses during repeated respiratory disease seasons must be accomplished before attempts at prevention of such illnesses can be fruitful.

Proposed Course of Project:

These studies which attempt to find new agents associated with adult respiratory illnesses will receive major emphasis in this laboratory as recent efforts to recover such agents by using human embryonic organ cultures and experimental cell cultures have been highly rewarding. In addition, the role of bacteria alone or in association with viruses in the etiology of respiratory disease will be studied more extensively.

Honors and Awards: None

Publications: None

Serial No. NIAID 70A
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Laboratory Studies of Myxoviruses and Paramyxoviruses Associated with Respiratory Tract Disease

Previous Serial Number: Same

Principal Investigators: Dr. Elias G. Chalhub
Dr. David Hodes
Dr. Brian Murphy
Miss Sandra Nusinoff
Dr. Robert M. Chanock

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (part B)	Dr. R. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	80/12			80/12
Professional:	44/12			44/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To produce conditional lethal temperature-sensitive (ts) mutants of myxoviruses and paramyxoviruses for possible use in live attenuated vaccines; (2) to study the genetic basis for attenuation shown by temperature sensitive mutants; (3) to define the relative importance of the various host defense mechanisms in resistance to myxovirus and paramyxovirus infection and disease.

Methods Employed:

A. Influenza - Influenza A virus suspensions were initially prepared in bovine kidney tissue culture. Mutagenesis of influenza A/HK/45/68 (H3N2) was accomplished by growth of this virus in BK roller tube cultures in the presence of media containing $10^{-2.5}$ M 5-fluorouracil. High titered suspensions of virus for use in volunteer studies and for genetic characterization of temperature

sensitive mutants were prepared by growing the bovine kidney passaged virus in the allantoic sac of either avian leukosis free eggs (for volunteer studies) or of regular embryonated eggs (for strains to be used for laboratory characterization).

Genetic analysis of temperature sensitive mutants was accomplished in two ways. In the plate complementation technique allantoic fluid suspensions of different mutant pairs were mixed, and inoculated onto monolayers of BK cells in petri dishes according to the usual plaque technique for the quantitation of influenza virus. These cultures were incubated at a temperature restrictive for plaque formation for each of the viruses individually. If plaques did develop the viruses inoculated were assumed to possess genetically distinct lesions, one virus providing for its partner in the cross the gene product (or products) which was defective in the other and thereby enabling plaque formation to occur. In the standard recombination and complementation tests BK roller tubes were doubly infected with pairs of mutants and then incubated (after absorption and washing) at restrictive or permissive temperature for 24 hours. Harvests taken after 24 hours were then titered at both permissive and restrictive temperatures. Analysis of the comparative titers of the 32°C harvests at 32°C and 39°C allowed calculation of the frequency of recombination. Evidence of plaque formation at 32°C by virus suspensions propagated at 39°C indicated that complementation and possibly recombination had occurred.

B. Parainfluenza Viruses - In genetic studies, parainfluenza 1, parainfluenza 2, and parainfluenza 3 viruses were grown in African green monkey kidney (AGMK) culture then purified by 3 successive terminal dilutions. Virus was then exposed to a chemical mutagen (5-fluorouracil) in the growth medium during viral replication. Clonal populations of the treated virus are then tested for capacity to grow at low or permissive temperature (30°-32°C) and at a high or restrictive temperature (38°-39°C). This evaluation was performed in 96-well Falcon microplates which contained trypsinized AGMK cells. A master plate was inoculated with a dilution of treated virus which was calculated to infect 10-20% of the wells. At this multiplicity of infection each infected well received only one infectious unit in the inoculum. After 5-6 days of growth the master plate was replicated into two additional plates with sterilized steel replicators. The master plate was then frozen at -70°C. One of the replicated plates was incubated at 32°C or the permissive temperature and the other at 39°C or restrictive temperature for 5-6 days. The replicated plates were then hemadsorbed with 0.1% guinea pig red blood cells. The viral populations in those wells which exhibited hemadsorption only at the permissive temperature represented the desired temperature-sensitive (ts) conditional lethal mutants. The appropriate wells were then identified in the frozen master plate and the provisional ts mutants were grown in AGMK cell cultures to produce suspensions of virus which are then evaluated in tissue cultures, experimental animals and volunteers for temperature sensitivity and attenuation.

Major Findings:

A. Influenza: Isolation and Characterization of Temperature Sensitive Recombinants - As described previously, a series of temperature sensitive recombinants of influenza A/Hong Kong/68 (H3N2) were derived by mixedly infecting BK tissue culture with wild type Hong Kong virus (H3N2) and temperature sensitive mutants of influenza A/Great Lakes/65 (H2N2). These virus strains were further analyzed on the basis of their efficiency of plaque formation over a range of temperatures from 32°C to 39°C. An unusual pattern of temperature sensitivity was observed for recombinants derived from the original 37°C shutoff H2N2 ts-1 parent. One class of recombinant shutoff at 37°C and thus resembled the ts-1 parent; another group of recombinants shutoff at 39°C while a third type of recombinant was markedly depressed in plaque formation at 38°C. Genetic evidence of two sorts now indicates that three distinct classes of recombinants were derived from the H2N2 ts-1 mutant and that these three classes are in turn distinct from those temperature sensitive recombinants derived from the H2N2 ts-2 parent virus.

Four prototype clones of ts recombinant mutants bearing the H3N2 configuration of surface antigens were propagated and cloned in BK tissue culture and passaged in eggs by the allantoic route of inoculation to yield high titered suspensions for genetic analysis. Each of these virus suspensions failed to produce plaques at the restrictive temperature of 39°C. If any of these strains were mixedly infected with another of the strains,however, plaque formation did occur. This plaque formation furthermore could be demonstrated to exhibit two-hit kinetics calculated on the basis of Poisson distribution and taking into account multiplicity of infection. Observed numbers of plaques were consistently 10-50 times greater than those predicted, while following dilution kinetics at the rate expected. This was taken to be evidence of the existence of considerable amounts of genetically active but non-infectious virus in the stocks used for these tests. The ts recombinants not only showed plaque complementation with the other H3N2 prototype strains, but also were shown to complement with the appropriate H2N2 ts parents.

The genetic distinctiveness of the prototype recombinant mutants was also demonstrated by performing the standard tests for evidence of recombination and complementation. When two mutants which possessed distinct ts lesions were grown together at 32°C (permissive temperature) for 24 hours and the resulting virus yield then titered at permissive and restrictive temperatures, recombinant virus which possessed the wild type phenotype, i.e., formed plaques at restrictive temperature, was demonstrated. Recombination frequencies varied from 0.5-10%. For some combinations of viruses, complementation was also shown when mixed infection was performed at restrictive temperature, but this was limited by our ability to grow very high titered suspensions of mutant virus. In all possible combinations of prototype ts mutant viruses, however, recombination to yield wild type progeny was demonstrable. By two techniques, therefore, the existence of four genetically distinct and non-overlapping classes of H3N2 recombinant virus was seen. Three of these classes of temperature sensitive influenza mutants have been evaluated in human volunteers, as indicated in

another section of this report, and a suspension of the prototype of the fourth class of recombinant viruses is presently being safety tested for administration to volunteers in the future.

In order to derive additional temperature sensitive mutants, i.e., mutants with different patterns of temperature sensitivity and lesions genetically distinct from those carried on the mutants already characterized in the laboratory, a wild type suspension of influenza A/Hong Kong/45/68 was directly mutagenized through growth in $10^{-2.5}$ M 5-fluorouracil. Approximately 1000 plaques were picked representing progeny from a virus suspension grown for 48 hours in the presence of the mutagen. From these 1000 plaques, fourteen clones exhibiting various patterns of temperature sensitivity have been derived. These are presently being plaque purified to insure genetic stability and homogeneity. After purification in bovine kidney culture these new mutants will be grown in eggs and then analyzed by the plate complementation assay to detect new lesions and/or to place the mutants in the four complementation groups presently established.

A wild type strain of influenza A/AA/Marton/43 (H0N1) obtained from the laboratory of F. Davenport in Ann Arbor, Michigan, is presently being adapted to the bovine kidney assay system in the laboratory. This virus, which has been cloned 3 times by plaque passage in BK cells, will be used in two different experimental designs. Its surface antigen configuration provides a new source of seronegative individuals in whom new temperature sensitive mutants of influenza can be evaluated since most persons born after 1947 lack antibody for the H0 hemagglutinin. Therefore, this strain will be used to accept temperature sensitive genes which have been shown to render influenza A (H2N2) and (H3N2) viruses attenuated for man. In this manner we may be able to determine the feasibility of using ts mutation and genetic recombination for the immunoprophylaxis of the anticipated H4 antigenic variant of influenza A virus.

The H0N1 strain of wild type virus will also be employed in recombination experiments with the four classes of recombinant viruses already genetically characterized to provide further information concerning the localization and number of lesions characteristic of each complementation group. As was the case in the H2N2 - H3N2 recombination experiment, this sort of analysis may permit the segregation of several lesions from a virus heretofore considered to contain only one lesion.

B. Production of Temperature Sensitive Mutants of Influenza B Virus - The acquisition of a ts defect by influenza A virus is associated with attenuation of these viruses for man. A suitable balance between loss of virulence and ability to stimulate an immunological response has been achieved using this method for attenuation. Thus, it seems reasonable to induce ts mutants of influenza B virus in an attempt to produce a live virus vaccine for this agent. Influenza B virus does not undergo the abrupt antigenic change characteristic of the influenza A viruses, but undergoes a more gradual type of change. With appropriate antisera distinctions can readily be made between certain influenza B viruses. Such a distinction exists for the influenza B/Bethesda/1971 and

influenza B/Maryland/1959 viruses thereby making it feasible to search for recombinants between these viruses using the appropriate antisera. We plan to produce ts mutants of influenza B/1959 by mutagenesis and to transfer the resulting ts lesions to influenza B/1971 via recombination. In our experience with influenza A viruses this process of recombination allows for the isolation of mutants with lesions on a single cistron whereas ts virus isolated from a pool of mutagenized virus frequently have lesions affecting several cistrons. Ts viruses with lesions in only one cistron may prove to be especially desirable because their altered gene can be passed with ease via recombination to new antigenic variants. If the altered gene specifies the desired degree of attenuation, then it is likely that the ts recombinant will exhibit this level of attenuation.

Influenza B/Maryland/1959 has been plaque purified three times and is ready for mutagenesis with 5-fluorouracil. In addition, influenza B/Bethesda/1971 has been plaque purified and an appropriate suspension of wild type virus for evaluation in volunteers is being prepared. It is planned to induce ts lesions in the 1959 virus and then transfer lesions by recombination to the 1971 virus.

Evaluation of the attenuation achieved by this process will be made by administering the most promising ts recombinant (as indicated by studies in hamsters) to volunteers and comparing their response with that observed following infection with wild type virus.

C. Parainfluenza I - Initial mutagenesis of the parent parainfluenza I pool yielded a mutant (ts-4) with a restrictive temperature of 38°C and decreased growth at 37°C. In vitro characterization of ts-4 in AGMK and rhesus MK tissue culture indicated that it was stable in vitro. However, after infecting hamsters with ts-4, the virus recovered from the lungs showed a high rate of reversion to wild type and thus was not acceptable as a potential vaccine strain.

The ts-4 parainfluenza I mutant was then remutagenized with 5-fluorouracil and additional mutants were sought in the manner described above. It was hoped that the resultant progeny would be more defective and consequently have a low reversion rate. Thus far six mutants have been identified with restrictive temperatures ranging from 37°-39°C. They are being characterized in vitro and are being studied in laboratory animals to evaluate their stability and immunogenic potential.

Efforts to obtain ts mutants of type 2 and type 3 virus by chemical mutagenesis with 5-fluorouracil have been unsuccessful thus far despite considerable effort.

D. Conditional Lethal Temperature Sensitive Mutants of Respiratory Syncytial Virus - Attempts to elucidate the nature of the temperature sensitive (ts) lesions of the existing mutants have continued. In addition, the production and evaluation of new mutants is under way.

Further analysis of the complementation groups of the existing mutants has been based on the "plaque complementation" technique. In this test, two different mutants, each incapable of producing plaques at the restrictive temperature,

are used to infect a cell layer. Infected cells are incubated at restrictive temperature and are subsequently examined for the presence of plaques. The development of plaques indicates that syncytium formation can be initiated at restrictive temperature when a single cell is doubly infected by two different mutants, each of which is incapable of producing syncytia alone. Such mutants complement one another and thus do not share a common genetic ts lesion. From the multiplicity of input of each of the mutants, the number of cells expected to be doubly infected can be determined. A comparison of this number with the number of plaques actually observed will give the efficiency of complementation.

Plaque complementation studies have confirmed some of the conclusions regarding complementation groups that had previously been established from growth studies. Ts-1, ts-3, and ts-4 (all produced with 5-FU) appear to belong to one group which is distinct from ts-7 (produced by Dr. Louis Potash, Flow Laboratories with another mutagen, ethylmethanesulfonate). Plaque complementation tests with ts-5 and ts-6 have been hampered by leakiness and reversion. Unfortunately, leakiness has also prevented plaque complementation study of ts-2, the mutant whose complementation group could not be determined from the growth studies. Further, the plaque morphology mutation previously attributed to this virus has been difficult to reproduce. It is also noteworthy that the pseudo-recombination phenomena observed with this mutant and attributed to complementing heterozygotes could not be reproduced. For this reason, new clonal populations of ts-2 are being derived by plaque isolation.

Three other mutants have recently been produced by Dr. Potash using proflavine sulfate. They have been designated ts-9, ts-10 and ts-11. A suspension of ts-10 has been prepared for plaque complementation studies. Ts-9 and ts-11 have so far proven genetically unstable.

Several other approaches to pinpointing the genetic lesions of each of the mutants have been attempted. Protein production by the mutants at restrictive temperature has been examined by fluorescent antibody and complement fixation (CF) techniques. It has previously been noted that ts-1, ts-2, and ts-3 each produced some degree of fluorescent stainable antigen (FA) at restrictive temperature whereas ts-4 produced none. These observations have been extended and refined. It is now apparent that ts-1, ts-3, and ts-10 are capable of producing FA at restrictive temperature. This antigen, however, is restricted to coarse chunks distributed within the cell cytoplasm. By contrast, at restrictive temperature, ts-7 produces FA which is distributed diffusely throughout both the cell membrane and the cytoplasm, a pattern resembling that seen with wild type virus.

Each of the mutants so far tested have produced some CF antigen at restrictive temperature with the exception of ts-1 and ts-4. The production of CF antigen by ts-7 and ts-10, however, is much reduced compared with the amount made at permissive temperature.

Another technique has been used to determine the phase of the replicative cycle in which the genetic lesions of the individuals' ts mutants are operative. In these experiments, mutants are adsorbed onto sheets of HeLa

cells and allowed to incubate at permissive temperature for the duration of the eclipse period (estimated at 12 hours). At the end of this period, the cells are shifted to restrictive temperature and incubation carried out for an additional 12 hours. At this time the cells are harvested, virus titered, and the titers compared with those of control cultures of the mutants which have been incubated at the permissive temperature for the entire 24 hour period. A decrease in titer in the temperature-shifted cultures, compared with the control cultures, suggests that the mutant has a lesion which is operative in the late portion of the replicative cycle. Studies to date indicate that ts-1 and ts-5 have lesions in genes which function in the later part of the cycle. Ts-6, ts-7, and ts-10, also show reduced titers in the temperature shift experiments, but their titers are not reduced as markedly as those of ts-1 and ts-5. Presumably their ts lesions affect functions occurring in the later stages of the replicative cycle but these functions are also operative, to some degree, at an earlier stage.

More recently, biochemical techniques have been employed to study the basic physiology of the parent RS virus and its mutants. Wild type RS virus is able to grow normally in HeLa cells in the presence of 0.01 ug-0.33 ug/cc actinomycin D. This same concentration of actinomycin D has caused 90% inhibition of the incorporation of tritium-labelled uridine into RNA by uninfected HeLa cells. Use of actinomycin D will enable us to initiate the following efforts:

(1) RS virus labelled with radioactive uridine can be prepared and can be utilized for radioimmunoprecipitation tests; (2) the existing ts mutants and wild type virus can be compared for their ability to make RNA at restrictive temperature; (3) the existing ts mutants and wild type virus can be compared as to their ability to incorporate radioactive amino acids, at restrictive temperature. All these investigations should provide data of a more quantitative nature than is currently available.

A final series of laboratory experiments is aimed at producing mutants with greater genetic stability than those so far isolated. When the current mutants are "stressed" by incubating infected cultures at restrictive temperature for 6 days, clones frequently emerge which have lost, or partially lost, their characteristic temperature sensitivity. It is likely that suppressor mutation or reversion to wild type has occurred and is responsible for the observed results. It is felt that inducing additional genetic lesions in the current mutants may make them more stable genetically. For this reason, the current vaccine strain of ts-1 has been "remutagenized" by exposure to ethylmethane sulfonate. We will now attempt to isolate clones from the remutagenized pool which are able to undergo a "stress test" without losing the original pattern of temperature sensitivity.

E. Pathogenesis of RS Virus Infection in Hamsters - Epidemiological observations suggest that some component of the severe RS virus bronchiolitis and pneumonia which occurs in young infants and in vaccinees with serum antibody to RS virus might be a result of immunologically mediated injury. It was hoped that by studying an experimental infection in hamsters that some of the factors involved in development of RS virus disease and in recovery from RS virus infection might

be defined. RS virus causes an inapparent infection in hamsters and virus replication occurs in nasal turbinates and the lungs but there is no evidence of tissue damage.

Experimental immunosuppression has been used to alter a number of viral infections so that the level of virus replication is increased and tissue damage occurs in what is otherwise an inapparent infection. For this reason we tested the effect of 2 immunosuppressive treatments: X-irradiation and cyclophorphamide. Unfortunately, neither treatment enhanced virus replication nor was tissue injury induced by these treatments. However, a definite delay in virus clearance from the nasal turbinates and lungs was seen and this correlated with delayed development of serum neutralizing and CF antibody. The effect of these treatments on interferon could not be assessed since interferon could not be detected during unmodified RS virus infection in the hamster. Also, both immunosuppressive techniques depressed perpheral white blood cell counts below normal. Therefore, we were unable to determine the relative importance of depressed immunological response per se in the observed delay in resolution of RS virus infection.

Further attempts to produce pathology in RS virus infected hamsters were undertaken by administering serum antibody prior to exposure to RS virus. Under these conditions, pathology might be produced if the virus were able to replicate and if an immunologically mediated injury were involved in the pathogenesis of RS disease. Three groups of animals were infected with RS virus: (1) a control group, (2) a group given RS virus given intranasally one month previously and (3) a group that received hamster hyperimmune RS virus antisera to yield a serum level comparable to group 2. Although RS virus grew well in group 3, no enhancement of pathology occurred. Virus replication was comparable in groups 1 and 3 but depressed in group 2. Of interest was the observation that animals in group 3 seemed to have a delayed and decreased development of serum antibody. One could speculate that perhaps the enhanced disease seen in infants, who possess passively acquired serum antibody, may result in part from impairment of their immune response to infection leading to enhanced viral replication. An experiment to determine if this observation is reproducible is in progress.

It is felt that this experimental RS infection in hamsters is an unsatisfactory model for pursuing questions concerning the role of immunological factors in the pathogenesis of RS virus disease because all modalities of experimental manipulation have failed to produce tissue damage in the hamster. Perhaps RS virus infection in another animal in which disease occurs naturally might help clarify the role of immunological factors in RS virus infection.

F. Development of Delayed Hypersensitivity to Parainfluenza 3 Virus in Guinea Pigs - The role of delayed hypersensitivity in recovery of animals from respiratory virus infections and to resistance to reinfection with these viruses remains to be defined. We have attempted to develop an animal model suitable for such an investigation. Parainfluenza 3 virus causes an inapparent infection in guinea pigs with production of serum antibody. Immunosuppressive treatment delays clearance of virus from the upper and lower respiratory tract

with a concomitant delay in appearance of serum antibody suggesting immunologic factors are required for resolution of infection. Currently, we are attempting to develop a technique for measuring local and systemic delayed hypersensitivity to determine whether these defense mechanisms are stimulated during infection and what effect immunosuppress therapy has on these responses. An assay of migration inhibitory factor has been developed and a dose response curve for DNP-BCG antigen has been established using systemic lymphocytes and peritoneal macrophages. However, we have failed to demonstrate delayed hypersensitivity to this antigen using respiratory tract lymphocytes. Attempts are underway to develop a parainfluenza 3 antigen suitable for use in the delayed hypersensitivity assay.

Significance to Biomedical Research and the Program of the Institute:

Since our initial recovery of parainfluenza viruses from man 13-15 years ago, clinical and epidemiological studies have established these agents as important pathogens of the respiratory tract. During primary infection with parainfluenza 1, the lower respiratory tract is often involved, and illness may be quite severe. Type 3 parainfluenza virus produces a spectrum of clinical illnesses in children including rhinitis, pharyngitis, bronchitis, croup, bronchiolitis, and bronchopneumonia. Thus, it is clear that immunoprophylaxis is needed to thwart the impact of these viruses in infants and young children. Inactivated parainfluenza virus vaccines have not provided significant resistance to respiratory illness caused by these viruses in infants and children. For this reason we have turned our efforts toward the development of live attenuated vaccines which will stimulate protective local immunity in the respiratory tract without causing disease.

Similarly, influenza A virus continues to be an important cause of respiratory tract disease morbidity and mortality although relatively effective protection can be provided by administration of a sufficient quantity of inactivated antigen by the parenteral route. In our view local respiratory tract immunity can be induced more efficiently by infection with an attenuated virus. In addition to this advantage an attenuated vaccine would probably be more readily accepted by the public and thus have a greater impact in prevention of disease. These theoretical advantages are sufficiently attractive for us to continue our efforts to develop mutants of influenza virus with the desired degree of attenuation and immunogenic activity.

In addition to demonstrating the feasibility of the ts approach to attenuation of influenza virus, several interesting observations concerning the nature of the ts mutations produced by 5-fluorouracil were made. We were able to identify 4 discrete ts lesions in the 2 mutants of influenza A/Great Lakes/65 (H2N2) which were studied most extensively. One of the mutants, ts-1, contained 3 separate lesions--one lesion imposed a temperature limitation of 37°C, while the others produced a limitation of growth at 38° and 39°C, respectively. The second mutant, ts-2, had a distinct lesion which also imposed a limitation of growth at 39°C. Ts recombinants with genetic lesions limited to one cistron should prove useful in future studies for identifying similar

or distinct lesions present in other mutants or recombinants. This will be particularly important when an altered gene, such at ts-1[E], is transferred by recombination to a new antigenic variant of influenza for the purpose of producing a new vaccine strain. In this circumstance the single lesion ts recombinants will be required to confirm by complementation analysis the identity of the lesion transferred to the new virus. Finally it should be mentioned that the existence of 3 distinct lesions (or clusters of lesions) in the ts-1 mutant did not become evident until recombination with the Hong Kong virus (H3N2) yielded 3 classes of recombinants which complemented each other.

Studies of the role of various host defense mechanisms in recovery from respiratory tract infection are clearly relevant to the Institute's program for control of this type of disease. A better understanding of the relative importance of the different defense systems should prove to be of inestimable value in planning the most effective means for control of respiratory tract illness.

Proposed Course of Project:

Study of the ts mutants of the parainfluenza viruses will continue in an effort to determine their potential for use in a live virus vaccine. Evaluation of the mutants for degree of defectiveness and attenuation will be conducted first in experimental animals and then in young adult men and subsequently in young children.

Additional genetic manipulation of the ts strains of influenza A presently available will be attempted with the aim of selecting new ts recombinants with the desired properties of attenuation and immunogenicity. The ts lesions will be transferred by recombination to influenza A (H0N1) in order to prepare strains which can be evaluated for attenuation in seronegative volunteers. (Individuals under 25 years of age lack antibody for the H0 hemagglutinin). This second transfer of ts lesions (originally induced in influenza A/Great Lakes/65 (H2N2) and then transferred to influenza A/Hong Kong/68 (H3N2))should also prove helpful in establishing whether or not the lesions which we now believe to affect only one cistron are indeed of this limited type.

Ts mutants of influenza B virus will be produced and these mutants will be manipulated in the same manner which proved to be successful with influenza A virus. Ts mutants or recombinants of influenza B virus will be investigated genetically and will also be evaluated in hamsters and subsequently in man for evidence of attenuation.

Additional ts mutants of RS virus will be sought and then compared by complementation analysis in an effort to obtain mutants with defects distinct from those already available. Attempts to define the stage in replication of RS virus at which different mutants are blocked will continue. Further, the ts-1 mutant will be remutagenized by exposure to EMS, a potent chemical mutagen, in an effort to introduce additional ts lesions into the virus. This will be done in an attempt to obtain a more stable mutant, i.e., one which will not

revert to wild type or exhibit pseudo-wild type properties as a result of a suppressor (or correction) mutation.

Honors and Awards: None

Publications: None

Serial No. NIAID 70B
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Classification and Characterization of Rhinovirus Serotypes of Human Origin

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: 8 Collaborating U.S. Laboratories and 2 WHO International Reference Centers (Common Cold Research Unit, Salisbury, Wilts, England, and Baylor College of Medicine, Houston, Texas

Contract Support

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	11/12			11/12
Professional:	7/12			7/12
Other:	4/12			4/12

Project Description:

Objectives: (1) To classify the numerous rhinovirus serotypes in order to develop a suitable nomenclature system; (2) to characterize rhinovirus serotypes antigenically; (3) to elucidate the interrelationships between different rhinovirus serotypes.

Methods Employed and Major Findings:

Rhinovirus Collaborative Program - The first and second phases of this collaborative program which was designed to characterize and enumerate the number of distinct rhinovirus serotypes and which was described in detail in previous annual reports instituted a new numbering system for rhinoviruses based upon extensive cross-testing of a large number of strains. The first phase of this numbering system enumerated 55 distinct serotypes from 1-55 with one subtype. The second phase added 34 additional distinct serotypes thereby extending the numbering system to include rhinoviruses 56 through 89. A third

phase of the program includes certain second phase viruses which demonstrated oneway or unresolved relationships and prototype candidate viruses distinct from previously numbered serotypes. It is anticipated that the third phase will be concluded during the next year.

Study of Rhinoviruses Recovered from Epidemiologic Studies - 171 acid labile, chloroform stable viruses have been recovered from patients enrolled in the NIH, LID Common Cold Study. These agents are classified as presumptive rhinoviruses on the basis of tissue culture characteristics and biochemical properties. With the completion of the second phase of the collaborative program and the availability of small amounts of antisera for all numbered serotypes, attempts are being made to type some of these rhinovirus isolates. Such a study will not only provide valuable information on the approximate number of uncharacterized serotypes, but also yield epidemiologic information on the prevalence of different serotypes in this community. Thus far, 23 acid labile, chloroform resistant viruses have been tested in neutralization tests with antisera for the 90 numbered rhinoviruses (i.e., 1-89 plus the subtype); 16 were typed (4 as rhinovirus [R] 2, two as R14, two as R41 and 8 as other different individual serotypes) and 7 could not be typed with the 90 available rhinovirus antisera. These seven untyped viruses are described in the two following sections of this report. Acid labile viruses obtained from studies at Junior Village are described in Project 66A.

Characterization of Two Newly Recognized Rhinovirus Serotypes of Human Origin - Two virus strains, 607 and 1169, were recovered from two volunteers in the NIH LID Common Cold Study. Strain 607 was isolated in HDCS WI26 and HDCS WI38 cultures and MA177 cell cultures from nasal washings obtained on 9/17/65 from a 28 year old male, and strain 1169 was recovered in semi-continuous human embryonic intestine (HEI) MA177, and semi-continuous HEI MA349 cell cultures, from nasal washings obtained on 9/25/70 from a 56 year old female. HEI MA349 is an additional HEI cell culture recently available from the laboratory of Mr. M. Vincent of Microbiological Associates, Inc. Since both virus strains induced a "rhinovirus-like" CPE, were acid labile and chloroform resistant, they were tested in neutralization tests with sera containing at least 20 antibody units of each of the 90 numbered rhinoviruses and were found to be distinct by the "oneway" neutralization tests. Strains 607 and 1169 were subsequently "purified" by 3 limiting dilution passages; guinea pig hyperimmune antisera was produced utilizing the "purified" virus as immunizing antigen. Strains 607 and 1169 were distinct from each other by reciprocal neutralization tests. In addition, 3-320 TCD_{50} of "purified" 607 and 1169 viruses were not neutralized by at least 20 antibody units of each of the 90 numbered rhinoviruses; 32-320 TCD_{50} of each of the 90 numbered rhinoviruses were not neutralized by 1:20 dilutions of virus 1169 guinea pig antiserum with a homologous titer of >1:5120 (vs 32 TCD_{50}); 32-320 TCD_{50} of 89 of the 90 numbered rhinoviruses were not neutralized by similar dilutions of virus 607 guinea pig antiserum with a homologous titer of 1:2560 (vs 100 TCD_{50}). Rhinovirus 87 was neutralized at low levels (but not by 20 antibody units) by rhinovirus strain 607 guinea pig antiserum. Such reciprocal neutralization tests established the serologic distinctness of these two rhinoviruses from the previously described 90 strains. "Purified" antigens of the two strains

were further characterized as follows: (1) each strain measured approximately 27 nm as determined by "immune electron microscopy"--a technique utilized so that the virus particles would be in the form of aggregates rather than indistinctive individual particles; (2) both strains had an RNA core as determined by the IUDR test; (3) both strains were acid labile and chloroform stable; (4) although both strains were relatively heat stable (at 50°C for 1 hour), they were both further stabilized under these conditions in the presence of magnesium chloride. In addition, paired sera from the two patients from whom these strains were recovered demonstrated significant neutralizing antibody rises against the homologous virus strain. Both strains are to be submitted to the third phase of the NIAID-WHO Collaborative Rhinovirus Program.

Continuing Characterization of Possible New Rhinovirus Serotypes - Five additional strains (604, 626, 715, 743 and 812) recovered from nasal washings obtained from volunteers in the NIH, LID Common Cold Study have not been neutralized by antisera to the 90 numbered rhinoviruses. Virus 604 was recovered in HDCS WI26 cultures, induced a "rhinovirus-like" CPE, was acid labile, chloroform stable and was not neutralized by sera containing at least 20 antibody units of each of the 90 numbered rhinoviruses and the NIH candidate prototypes 607 and 1169. Virus 604 has been "purified" by 3 limiting dilution passages and guinea pig antisera has been produced from "purified" antigens. Further characterization is in progress.

Strain 626 was recovered in HDCS WI26 and HDCS WI38 cultures and AT39 and HEI MA177 cell cultures, strain 715 in HDCS WI26 and HDCS WI38 cultures and HEI MA177 cell cultures, and strain 743 in HEI MA177 cell cultures; strain 812 was not recoverable in the usual tissue cultures utilized in this laboratory and therefore was passaged 3 times into human embryonic tracheal organ culture (OC); such OC harvests produced CPE in both HEI MA177 cell and HDCS WI38 cultures. All four strains induced a "rhinovirus-like"CPE, were acid labile and chloroform stable and were not neutralized by antisera containing at least 20 antibody units to the 90 numbered rhinoviruses. Further characterization of these viruses is in progress.

Significance to Biomedical Research and the Program of the Institute:

Rhinoviruses are the most important known group of viruses to be associated with acute upper respiratory tract illnesses in adults. However, the rapidly increasing number of serotypes and the absence of an acceptable numbering system made interpretation of epidemiological data difficult and also prevented satisfactory epidemiological investigations of rhinovirus infections. The numbering system will permit such studies to be undertaken satisfactorily and such studies will be important in further evaluating the need for rhinovirus vaccines.

Proposed Course of Project:

It is anticipated that the third phase of the collaborative program will include significantly fewer viruses than in either the first or second phases,

and this phase should be completed during the next year. In addition, studies on the different antigenic components of individual rhinovirus serotypes will be expanded using the technique of "immune electron microscopy" described in detail in last year's annual report. For this purpose R1A antiserum has been prepared in guinea pigs utilizing both heat inactivated R1A antigen and uninactivated antigen; in addition, the effects on viral aggregation by different immunoglobulin groups, such as IgA, will also be investigated.

Honors and Awards: None

Publications:

Kapikian, A.Z.: Chapter on Rhinoviruses. In Strains of Human Viruses, In press, 1972.

Serial No. NIAID 70C
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Mycoplasmas

Previous Serial No.: Same

Principal Investigators: Dr. Helmut Brunner
Dr. Robert Chanock

Other Investigators: Mr. Anthony Kalica (LID)
Dr. Benjamin Prescott (LM)
Dr. Itzak Kahane (NIAMD, LEP)

Cooperating Units: None

Contract Support: None

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	44/12		12/12	56/12
Professional:	20/12		12/12	32/12
Other:	24/12			24/12

Project Description:

Objectives: (1) To study the biological and biophysical properties of mycoplasmas, with particular emphasis on Mycoplasma pneumoniae, the etiologic agent of cold agglutinin positive pneumonia, and to determine the relationship of those properties to virulence; (2) to develop more sensitive and specific serologic techniques for the measurement of antibody to mycoplasmas; (3) to determine whether local antibodies develop in nasal secretions after infection; (4) to test the possibility of using hybrid reaggregates of the serologically active lipids of M. pneumoniae and membrane proteins of A. laidlawii as an immunizing agent in the hamsters model;(5) to study the infectivity, stability and protective capacity of ts mutants of M. pneumoniae (strain PI 1428) in the hamster animal model; (6) to study the infectivity and virulence of M. pneumoniae in young guinea pigs in order to obtain an animal model for the investigation of cell mediated immune response to the organisms; (7) to purify protein antigens from M. pneumoniae membranes and study their biochemical characteristics and serologic activities.

Methods Employed:

(1) Mycoplasmas were grown on agar plates or in broth suspensions. M. pneumoniae was also grown in Povitsky bottles under conditions in which the colonies became attached to the glass surface.

(2) The complement mediated billing reaction first described by Gale and Kenny for M. pneumoniae was made more sensitive by incubating organisms in the presence of serum for 60 minutes at 4°C prior to the addition of fresh guinea pig serum which served as the source of complement. In the modified killing or mycoplasmacidal test the decrease in viability of M. pneumoniae produced by various concentrations of antiserum was determined in the presence of a constant amount of fresh guinea pig serum, and the highest dilution of test serum which produced a 90% killing effect was estimated after logit transformation using a computer program.

(3) A radioimmunoprecipitation method which employed small quantities of reagents (0.025 ml) and microtiter equipment was developed. The organisms were grown in 2 liter Povitsky bottles in the presence of C^{14}-oleic acid and C^{14}-palmitic acid. The cell sheet adhering to the glass surface was washed 3-4 times after removal of the medium. These organisms were filtered through a 450 nm millipore filter and diluted to an activity of 500-1000 cpm per 0.025 ml. Radiolabelled antigen was incubated with different serum concentrations for 60 minutes at 37°C and then overnight at 4°C. The antigen-antibody complexes were precipitated by anti-human globulin produced in guinea pigs. After centrifugation of the precipitates at 1000 rpm for 10 minutes, the radioactivitity remaining in the supernatant was determined. From this determination the highest serum dilution which gave a 33% binding of antigen was calculated and considered to be the titer.

(4) Syrian hamsters were inoculated intranasally with either temperature sensitive (ts) or wild type M. pneumoniae. Response of the animals to infection was studied by titration of the number of organisms which had grown in the lungs and nasal turbinates, appearance of MI, CF and mycoplasmacidal antibody in the serum, and lung histopathology. Six weeks after initial infection, the animals were challenged intranasally with a virulent wild type M. pneumoniae strain and the response to challenge studied as above.

(5) Syrian hamsters were immunized with reaggregates of M. pneumoniae lipids and A. laidlawii membrane protein. Six weeks later the animals were challenged with wild type M. pneumoniae and the extent of infection and histopathology determined as described in 4.

(6) Weanling guinea pigs were inoculated intranasally with M. pneumoniae organisms. Response of the animals to infection was measured by titration of the organisms which had grown in the lungs, appearance of CF, MI and mycoplasmacidal antibody in the serum and lung histopathology. Transformation of splenic lymphocytes in the presence of heat killed M. pneumoniae organisms was measured by uptake of H3-thymidine in vitro.

(7) Weanling guinea pigs were injected intradermally with M. pneumoniae and the transformation of splenic lymphocytes to blast cells measured by uptake of H^3-thymidine.

Major Findings:

A. More Sensitive Serologic Techniques for Measurement of Antibody to M. pneumoniae - At present, metabolic inhibition (MI) and complement fixation (CF) are the most widely used methods for serodiagnosis of M. pneumoniae infections, but methods with increased sensitivity for detection of antibody are needed to illuminate certain unexplained aspects of M. pneumoniae infection. For example, there is the unusual age distribution of disease which occurs with greatest incidence in the 5-15 years age group. Then, there is the recent experience at Children's Hospital of D.C. in which an unexpectedly high frequency of shedding of M. pneumoniae was observed in young children who were not ill. Taken together, these findings suggest that M. pneumoniae disease might result from reinfection of individuals previously sensitized to the organism. In order to explore this possibility, highly sensitive methods for assay of M. pneumoniae antibody are required.

Investigation of the conditions which affect the mycoplasmacidal action of antibody and complement upon M. pneumoniae indicated the importance of a filtered suspension of predominantly single organisms, and an appropriate concentration of cations. The mycoplasmacidal effect of antibody and complement resembled the hemolytic reaction in that variation in concentration of either antibody or complement yielded an S-shaped curve. The antigens involved in the mycoplasmacidal reaction appeared to be the membrane glycolipids which are also of major importance in the complement fixation and metabolism inhibition reactions. The standardized mycoplasmacidal antibody assay proved to be considerably more sensitive than the conventional assay systems for measurement of M. pneumoniae antibody in human serum.

Both mycoplasmacidal (MC) and radioimmunoprecipitation (RIP) tests were used to study paired sera of volunteers collected prior to and 28 days after challenge with M. pneumoniae, strain PI 898. The mycoplasmacidal and RIP techniques were considerably more sensitive for detection of antibody to M. pneumoniae than the previously employed methods. Furthermore, the mycoplasmacidal and RIP methods measured large increments in antibody during convalescence from experimental infection. There was good agreement when the results of the two methods were compared with one another. The radioimmunoprecipitation method appeared to be slightly more sensitive than the mycoplasmacidal technique. It is quite clear that these tests are considerably more sensitive than the metabolic inhibition test. In addition, antibody was measurable by the mycoplasmacidal test when indirect immunofluorescence, metabolic inhibition and complement fixation (even employing highly specific membrane glycolipids as antigen) failed to detect antibody. A significantly higher mycoplasmacidal antibody titer ($p < 0.05$) was observed in pre-challenge sera of volunteers who did not become ill as compared to those who developed illness, indicating that this antibody was related to resistance to M. pneumoniae disease.

We then tested our hypothesis that M. pneumoniae disease might be caused by reinfection of individuals who had previous contact with the organism. First we studied a group of 7-12 month old children. In this group, maternal antibody should have disappeared and indeed we found that most sera were free of antibody. However, in the 13-36 month age group a large proportion of sera were positive by both methods, and the frequency of sera containing antibody increased with increasing age. The difference in frequency of antibody between the 7-12 month old children and the older age groups was statistically significant.

A decrease in frequency of detectable antibody in the 37-48 and the 49-60 month old children was seen and may be due to inadequate sampling or these children may lose antibody which then makes them more susceptible to M. pneumoniae infection.

All adults had RIP and almost all had MC antibody. The titers in adult sera was found to be significantly higher than those in children. The frequency of MI antibody increased with age but the titers were very low.

If MC and RIP measure specific antibody then a considerable amount of infection occurs early in life when M. pneumoniae disease is rare. These findings suggested that M. pneumoniae disease, which occurs with highest frequency between 5-15 years of age, may be a sensitization disease in which a prior silent infection prepares an individual for illness during subsequent infection.

The possibility that M. pneumoniae infection might be a sensitization disease was examined further by testing pre-infection sera from individuals who subsequently developed M. pneumoniae pneumonia. Sera collected from Marine recruits when they entered their training at Parris Island were assayed for MC and RIP antibodies. Essentially every man who developed M. pneumoniae pneumonia had RIP antibody before exposure to the organism. Mean MC and RIP antibody titers in pre-exposure sera of pneumonia patients were lower than those seen in a random sample of the Marine recruit population. One could speculate that lower levels of antibody in men who later developed pneumonia were a reflection of their susceptibility to the organism; however this is clearly speculation.

Local antibody in nasal secretions. We were also interested whether or not local antibody in respiratory tract secretions might play an important role in resistance to disease. Previous attempts to detect antibody in local secretions had failed, but this failure may have resulted from an insensitivity of the immunologic methods used. We recently studied nasal secretions from infected volunteers using the MC method and were unable to detect development of complement dependent killing antibody. However, using the RIP test it was possible to detect significant rises in local antibody in 9 of 13 infected volunteers. Overall there was a 23-fold increase in geometric mean RIP antibody titer in secretions by the 4th week after experimental infection. A total of 60 secretions from 13 men were tested and the results corrected for

10 mg% IgA. Since cidal antibody does not seem to be produced locally it may well be that the antibody which we measured is similar to that recently described by Jones for M. pulmonis. This antibody was shown to be opsonic and was required for the clearing of M. pulmonis by mouse peritoneal macrophages.

B. Mechanism of Antibody Mediated Killing of M. pneumoniae - The cell killing reaction produced by M. pneumoniae antiserum was shown to be mediated by antibody specific for the glycolipid hapten of the organism. In the presence of complement the organism was killed by a rabbit antiserum which contained antibody for the glycolipid hapten but not for the other constituents of M. pneumoniae. Complement was essential for killing of the organism. Apparently, viability was destroyed by lysis of this mycoplasmal membrane affected by antibody and complement operating in concert. This reaction was visualized by negative staining of antibody--complement treated organisms. When viewed under the electron microscope the affected organisms were seen to change from filamentous structures to large circular structures with many tears or holes in the limiting membrane. Additional evidence that the membrane had been compromised was provided by the observation that complement-mediating killing was associated with release of 260 nm absorbing material, presumably nucleic acid.

Small circumscribed circular lesions of 90-100 Å in diameter were seen in negatively stained preparations of M. pneumoniae organisms which were treated with antibody and human complement for a short period of time. Apparently the 90-100 Å holes in the membrane are the initial lesions and these are followed by more extensive holes or tears in the membrane. This is in good agreement with observations on the effect of antibody and complement on red blood cells, Krebs ascites tumor cells, bacterial and artificial membranes.

C. Temperature Sensitive Mutants of M. pneumoniae - In previous studies the ts mutants which were produced by treatment with NTG were stable during growth in broth and in the hamster lung. In the previous experiments the presence of revertant organisms in the lungs were sought in suspensions which had been stored in the frozen state. Recently we found to our surprise that mutants we had previously thought to be stable in vivo when tested by this procedure did indeed yield revertants when lung suspensions were tested fresh without prior freezing. This prompted us to re-examine the question of reversion and genetic stability. Seven of the mutants which were examined recently were found to be stable genetically when the more stringent procedure of testing fresh lung suspension was employed. Five of the mutants did not produce colonies at 37°C i.e., the mutants had a shut-off temperature of 37°C, while the remaining two mutants, H9 and H10, shut off at 38°C. In contrast, wild type organ grew as well at 38°C as at 32°C.

These 7 mutants were then studied in the hamster. The mutants and the wild type each were inoculated into a group of 35 hamsters. Five hamsters of each group were sacrificed immediately to determine the number of M. pneumoniae organisms present in the lungs shortly after inoculation. A group of 10

hamsters were sacrificed at day 10 and another group of 10 at day 40 after inoculation.

Lung suspensions were prepared and tested immediately on agar medium in order to quantitate the number of organisms present in the tissue. On day 42, the remaining animals (at least 10 animals per group) were challenged with 10^6 CFU of wild type organisms intranasally. Ten days later or 52 days after the start of the experiment these animals were sacrificed and their lung tissue tested for the quantity of organisms present.

H43, H28, 710, 640 and A4 which shut off at 37°C were not recovered from the lungs 10 days after initial administration. In contrast, H9 and H10, mutants which shut of at 38°C, grew to as high a titer in the lungs as the wild type strain. Significantly, all the organisms recovered from the lungs of hamsters given the H9 or H10 mutants had the ts phenotype, i.e., they produced colonies at 32°C but not at 38°C. It was of interest that 40 days after inoculation the H9 and H10 mutants were still found in relative high titer in pulmonary tissue. Again all the organisms recovered were temperature sensitive indicating that these mutants were stable genetically in vivo. When the hamsters which received the various mutants were challenged with wild type organisms (10^6 CFU) on day 42 and sacrificed on day 52, it was found that the H9 and H10 mutants as well as the 640 mutant had induced a significant resistance to the replication of the wild type challenge.

The lungs of the animals which were sacrificed were studied histologically. As expected the wild type organism caused severe lesions which were apparent 10 days after infection. Significantly the H9 and H10 mutants which replicated to a high level in the lungs did not cause appreciable lung lesions. On day 40 when moderately large quantities of H9 and H10 were still present in the lungs, lung lesions were not observed. It is evident from the examination of the mean lung lesion scores of hamsters sacrificed 10 days after challenge with wild type organism that prior infection with the 2 38°C shut-off mutants, H9 and H10, induced significant resistance to the disease producing potential of the wild type organism. Mutant 640 and to a lesser degree mutant 710 also induced measurable resistance to wild type organisms. The A4 mutant appeared to be completely ineffective in altering the effect of wild type challenge. Because of these encouraging results we are currently preparing suspensions of H9, H10 and 640 for evaluation in volunteers.

Significance to Biomedical Research and the Program of the Institute:

More sensitive serologic procedures for detection of M. pneumoniae antibody should prove helpful in illuminating several of the unexplained aspects of the biology of this organism. For example, it should be possible to obtain a definite answer to the question of local antibody response. This is particularly important since previous studies using less sensitive serologic methods failed to identify local antibody, although challenge experiments in hamsters have demonstrated that local respiratory tract immunity plays a major

role in resistance to M. pneumoniae. The possibility that prior silent infection with the organism sensitizes the host so that infection at a later date results in illness can now be investigated by the highly sensitive mycoplasmacidal and radioimmunoprecipitation antibody techniques. This represents a matter of some concern and has relevance to the direction to be taken in immunoprophylaxis of M. pneumoniae disease.

The development and characterization of ts mutants of M. pneumoniae appears to be one of the most promising approaches to prevention of disease caused by this organism. Current findings in this area offer some hope that the ts mutants will prove useful in the control of M. pneumoniae illness

Proposed Course of Project:

The sensitive mycoplasmacidal antibody and radioimmunoprecipitation techniques will be used to study sera from infants and children in order to confirm the recent findings which suggest that silent infection with M. pneumoniae is a frequent occurrence during early life. The specificity of the antibodies in such sera will be studied by absorption with specific membrane glycolipids of M. pneumoniae. The new serological tests for M. pneumoniae will also be applied to a further study of the local respiratory tract secretory antibody response.

Work with the ts mutants of M. pneumoniae will continue and will be concerned primarily with persistence of mutants in the lungs of infected hamsters. When this issue is resolved the mutant (or mutants) with the most desirable properties of a vaccine strain will be evaluated in adult volunteers for evidence of attenuation and immunogenicity.

Honors and Awards: None

Publications:

Brunner, H., James, W.D., Horswood, R.L., and Chanock, R.M.: Measurement of Mycoplasma pneumoniae Mycoplasmacidal Antibody in Human Serum. J. Immunol., In press, 1972.

Brunner, H., Horswood, R.L., and Chanock, R.M.: More Sensitive Methods for Detection of Antibody to M. pneumoniae. Proceedings Workshop on "Mycoplasmatales as Agents of Disease", J. Infect. Dis., In press, 1972.

Cole, R.M., Popkin, T.J., Prescott, B., Chanock, R.M., and Razin, S.: Electron Microscopy of Solubilized Acholeplasma laidlawii membrane proteins reaggregated with Mycoplasma pneumoniae Glycolipids. Biochim. Biophys. Acta 233: 76-83, 1971.

Razin, S., Chanock, R.M., Graf, L., and Rapport, M.M.: Immunogenicity of Cytolipin H Aggregated with Acholemplasma laidlawii Membrane Proteins. Proc. Soc. Exp. Biol. Med. 138: 404-407, 1971.

Steinberg, P., Horswood, R.L., Brunner, H., and Chanock, R.M.: Temperature-sensitive Mutants of Mycoplasma pneumoniae. II. Response of Hamsters. J. Infect. Dis. 123: 179-187, 1971

Serial No. NIAID 70D
1. Infectious Diseases
2. Respiratory Viruses
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Laboratory and Epidemiologic Studies of Viral Hepatitis Agents

Previous Serial Number: Same

Principal Investigator: Dr. Robert H. Purcell

Other Investigator: Dr. Stephen M. Feinstone

Cooperating Units:
Dr. Andrew Morrow (Surgery Branch, NHI, NIH)
Drs. Paul Holland, Harvey Alter and Paul Schmidt (Blood Bank, NIH)
Dr. John L. Gerin (MAN Laboratory)
Dr. William London (NINDS)
Dr. Morris Suggs (CDC)
Dr. James Maynard (CDC)
Dr. Charles Cherubin (Harlem Hospital)
Dr. Lloyd Roseboom (Johns Hopkins)
Dr. C.J. Peters (MARU)
Dr. W. Reeves (MARU)
Dr. John Fox (Univ. Wash., Seattle)
Dr. James Sgouris (Michigan Department of Health)
Dr. T. Edgington (Scripps)
Dr. Hyman Zimmerman (Washington V.A.)
Dr. Thomas Chalmers (CC, NIH)
Dr. David Madden (NINDS)
Dr. Dale Dietzman (NINDS)

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
NIH 71-2097	Dr. Purcell	Mel Labs. Inc.	Dr. Verna	$ 157,000
Interagency Agreement	Dr. Bryne	Oak Ridge Nat. Labs.	Drs. J. Liverman N. Anderson	326,000
Interagency Agreement	Dr. Bryne	CDC	Dr. M. Suggs	15,600
NIH 71-2197	Dr. Purcell Dr. Dunnick	Tulane Univ.	Dr. P. Gerone	48,565

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	60/12			
Professional:	24/12	3/12		60/12
Other:	36/12		4/12	27/12
				40/12

Project Description

Objectives: (1) To collect clinical material of high potential infectivity for use in laboratory studies; (2) to develop and test in vitro procedures and animal systems for use in laboratory studies of viral hepatitis; (3) to study the biochemistry, biophysics, immunology and epidemiology of hepatitis-associated antigen.

Methods Employed:

(1) Clinical materials were collected from patients undergoing major heart surgery at NIH and from various animal and human populations through collaborative studies as described below.

(2) Hepatitis B antigen (HB Ag) was purified by density banding in a cesium chloride gradient and by rate centrifugation in sucrose. Characterization was achieved with appropriate techniques, including analytical centrifugation, acrylamide gel electrophoresis and electrofocussing.

(3) Serum containing HB Ag was inoculated into various primate species and the animals monitored for evidence of hepatitis for six months after inoculation.

(4) Serologic techniques, including complement fixation, agar gel diffusion, counterelectrophoresis, immunoelectrophoresis and radioimmunoassay were used for serologic studies.

Major Findings:

(1) Hepatitis B Antigen - An abnormal protein, hepatitis B antigen, was found in the serum of a high proportion of persons with leukemia, Down's syndrome and hepatitis and was found to be specifically related to infection with the virus of serum hepatitis. Furthermore, the partially-purified antigen was associated with virus-like particles 20-25 nm in diameter. HAA was believed by some to be the virus of hepatitis. More recently, other morphological forms associated with HB Ag have been found, including a 43 nm complex particle (the Dane particle). This particle may be the virus of hepatitis B; much of the work of this project is being directed toward the study of the various forms of HB Ag.

(2) Serology (radioimmunoassay) - Recent controlled studies suggest that most of the currently available radioimmunoassay tests for detecting HB Ag sacrifice reproducibility for sensitivity. One new technique, however, a "sandwich" type radioimmunoassay for the antigen developed by Ling and Overby, proved to be both specific and sensitive. We are currently attempting to modify this technique to the microtiter format for the identification of subtypes of HB Ag.

(3) Epidemiology -

A. Longitudinal studies of transfused patients: Longitudinal studies of patients undergoing cardiac injury with massive transfusion at NIH were begun in 1964. These studies, described in detail in previous annual reports, yielded important information on the frequency of icteric and anicteric hepatitis as well as the relative importance of hepatitis B virus, cytomegalovirus, and EB virus in the etiology of transfusion-associated hepatitis. Furthermore, a strong association between the use of commercially derived blood and hepatitis in recipients of that blood was documented on the basis of these studies, modified blood banking procedures were initiated at the NIH Clinical Center Blood Bank, including pre-screening of all blood for the presence of HB Ag and the use of blood derived solely from non-commerical sources. The implementation of these changes in blood banking procedures resulted in a 90% decrease in the frequency of post-transfusion hepatitis among patients undergoing corrective cardiac surgery with massive transfusion at the NIH. Residual hepatitis among blood recipients appeared to have two causes: HB Ag-contaminated blood in which the antigen was undetectable or could be detected only with the most sensitive radioimmunoassay techniques, and blood contaminated with an agent causing HB Ag negative hepatitis with short incubation period, presumably hepatitis A. Studies of these patients who developed HB Ag negative hepatitis are in progress in an attempt to identify the agent.

Because of the diminishing frequency of hepatitis among blood recipients at the NIH, it is no longer practical to carry out longitudinal studies; for this reason, this phase of our hepatitis study has been discontinued.

B. Antibody to HB Ag in human populations: Previous studies, reported by us, revealed that antibody to HB Ag (anti-HB Ag), as measured by RIP techniques, was found infrequently in infants and children but in increasing frequency in young and middle aged adults (almost 40% of middle aged adults in a low socioeconomic group population had such antibody). The frequency of anti-HB Ag diminished slightly in the elderly. Such a finding suggested that there was a high sub-clinical attack rate of HB Ag-positive hepatitis in the general population. Our finding of antibody predominantly in adults is in good agreement with other studies in which HB Ag was found much more frequently in adults than in children. It seemed unlikely that such a high attack rate could be explained solely by parenteral spread of the virus; it is therefore probable that some other means of spread is operable.

To further characterize the exposure of HB Ag in various populations, we have performed seroepidemiologic studies on several populations in collaboration with other scientists. In collaboration with Dr. Charles Cherubin (New York),

we have studied the seroepidemiology of anti-HB Ag in a low socioeconomic group (Harlem, New York), a middle socioeconomic group (a Staten Island working class population) and a high socioeconomic group (Park Avenue, New York). In all three populations, the pattern of antibody acquisition (increasing frequency of antibody with increasing age) was found to be similar but the frequency of antibody in a given age group was found to be inversely related to the socioeconomic status of the population. Thus, infection with the agent of hepatitis B appeared to be most prevalent in low socioeconomic groups. The pattern of exposure, however, appears to be quite different from that of certain viruses spread by the fecal-oral route, such as poliovirus: infection of infants and children with hepatitis B virus appears to be rare in open populations. It should be noted, however, that failure of infants and young children to be infected with hepatitis B virus is probably not related to lack of susceptibility in these age groups, since children in closed populations, such as homes for the mentally defective, demonstrate a high rate of infection.

Further studies to delineate the epidemiology of hepatitis B in open populations are in progress. One such study is being carried out in collaboration with Dr. John Fox of Seattle. Families enrolled for several years in Dr. Fox's family studies are being tested for anti-HB Ag. Families with serologic evidence of infection with hepatitis B virus will be studied retrospectively in an attempt to determine the frequency of intra-family spread of the agent as well as the spectrum of clinical forms with which it is associated. In another collaborative study, with Drs. W. Reeves and C.J. Peters of MARU, family contacts of index patients with HB Ag-positive or HB Ag-negative hepatitis are being followed longitudinally in an attempt to determine the frequency of apparent and inapparent infection among family members. Preliminary evidence from these studies, still in progress, suggests that infection of family members,usually in an inapparent form, does occur.

C. Study of the risk of hepatitis to medical personnel: In a collaborative study with the CCBB and Dr. Thomas Chalmers (CC), individuals considered at risk for infection with hepatitis (physicians, nurses, clinical chemistry and pathology laboratory personnel, etc.) and a carefully matched control population of individuals not exposed to patients are being tested for HB Ag, anti-HB Ag and transaminase elevations. This study, performed under code, is still in progress. The goal of the study is to determine whether medical personnel are at significantly greater risk of contracting clinical or sub-clinical hepatitis than individuals not involved in patient care.

D. Seroepidemiology of HB Ag in a closed population: In a collaborative study with Drs. David Madden and Dale Dietzman (NINDS), the frequency of HB Ag and anti-HB Ag among residents of an institution for the mentally retarded was examined. There was found to be a marked variation in the frequency of antigen carriers from ward to ward but a striking direct relationship between frequency of antigen carriers and the frequency of anti-HB Ag among wardmates of the carriers on the various wards. These data add further confirmation to the belief that the detection of anti-HB Ag is an accurate measure of prior exposure to HB Ag. In contrast to results obtained from "open" populations,

anti-HB Ag was acquired at an early age: over 50% of children 6-10 years of age had detectable antibody and over 80% of children in the 11-15 year age group had such antibody.

E. Seroepidemiology of HB Ag in primitive populations: In collaboration with Drs. W.J. Reeves and C.J. Peters (MARU), we are studying the relative frequency of HB Ag and anti-HB Ag among three primitive peoples of Panama: the Choco, the Guaymi, and the Cuna Indians. Preliminary evidence suggests that the age-specific frequency of HB Ag and anti-HB Ag vary from population to population. Studies are in progress to determine if these differences are related to genetic or cultural differences among the tribes or to differences in the subtype of HB Ag endemic in the tribes. The age-specific acquisition of anti-HB Ag in these primitive populations was similar to that observed in a low socioeconomic group in the United States (Harlem, New York). Thus, infection with hepatitis virus appeared to occur most frequently in the older child and young adult.

F. Seroepidemiology of HB Ag in sub-human primates: Sera from over 1500 old world sub-human primates representing the major taxonomic classifications of these animals, have been studied for anti-HB Ag. Such antibody has been found in a variable proportion of such animals. In general, there was a direct relationship between the frequency of anti-HB Ag and the evolutionary status of the species tested. Thus, approximately 50% of chimpanzees tested had evidence of prior infection with the hepatitis B virus whereas less than 5% of macaques had such antibody. Similar studies on new world sub-human primates are currently in progress; preliminary results suggest that these animals are also susceptible to infection with the agent of hepatitis B.

G. Failure to detect a relationship between serum hepatitis infection of mothers and mongolism in their offspring: In a collaborative study with Drs. Dale Dietzman and David Madden, a retrospective seroepidemiologic study of mongoloid children and their mothers, and a carefully matched control group of normal children and their mothers, were studied for evidence of an etiologic association between hepatitis B infection and mongolism. No such association was found. Previous reports by others have suggested that such an association might exist, but our data strongly suggest that, if the association does exist, it is between hepatitis A and mongolism.

(4) Distribution of HAA in Fractions of Blood Fractionated by the Cohn Procedure - In a collaborative study with Dr. James Sgouris, Michigan Department of Health, and Dr. Paul Holland, Clinical Center Blood Bank, units of blood containing HB Ag, anti-HB Ag or anti-complementary activity, as well as blood from normal individuals free of HB Ag, anti-HB Ag or AC activity, were fractionated by the Cohn procedure, and the resultant fractions tested for HB Ag and anti-HB Ag by agar gel diffusion, counter-electrophoresis, complement fixation, and several radioimmunoassay methods. It was found that HB-Ag could be detected in all fractions, with the possible exception of fraction 2 (gamma globulin). The various radioimmunoassay procedures were found to yield false positive results when used for testing gamma globulin for the presence of HB Ag. Albumen, which is not infectious for hepatitis, contained HB Ag. Thus, the

infectivity of HB Ag-positive plasma could be separated from HB Ag, or alternatively, the hepatitis B virus was inactivated by the fractionation procedure.

(5) Antigenic Subtypes of HB Ag - It has now been confirmed in several laboratories that 2 and probably more antigenic specificities of HB Ag exist. The 2 best characterized specificities, subtypes ad and ay, appear to have epidemiologic importance; subtype ay is found more frequently in association with acute clinical hepatitis whereas subtype ad is found more frequently among inapparent HB Ag carriers and among those with chronic hepatitis. Confirmation of this preliminary finding must await the completion of appropriate epidemiologic studies and the development of more sensitive techniques for the subtyping of HB Ag.

Progress in the development of more sensitive subtyping techniques has been made in a collaborative study with Dr. Paul Holland of the CCBB. Cross-absorbed antisera specific for the subtype specificities (d and y) have been prepared and utilized in a sensitive counter-electrophoresis technique for the subtyping of HB Ag. In collaboration with the American Red Cross, a survey of HB Ag subtypes encountered in American Red Cross Blood Banks throughout the country is being carried out. Preliminary evidence suggests that there may be regional differences in the relative frequencies of the ad and ay subtypes of HB Ag.

In related studies, we are developing a modification of the radioimmunoassay technique for the specific subtyping of minute quantities of HB Ag. Preliminary results of these studies are encouraging.

(6) Preparation of Reference Reagents for HB Ag Subtypes - In collaboration with Dr. John Gerin (MAN) and Dr. Morris Suggs, (Biological Reagents Branch, CDC) large quantities of reference reagent quality anti-HB Ag were prepared in guinea pigs. This reagent was placed in the NIAID research resources repository. It has been the most frequently requested reagent in the RRB catalogue during the past year.

Subsequent studies indicated that the RRB anti-HB Ag reagent contained antibody directed against the a and d antigenic specificities of HB Ag. We have subsequently prepared guinea pig anti-HB Ag directed against the ay subtype of HB Ag; this reagent has been completed and is in the final stages of testing. We are currently determining the feasibility of preparing, by cross-absorption procedures using solid immunoabsorbants, specific anti-d and anti-y antibodies for subsequent release by the RRB.

(7) Other Antigenic Specificities Associated with HB Ag - Recently Almeida demonstrated that the Dane particle, which shares antigenic specificity with HB Ag, contained a virus-like internal component antigenically distinct from HB Ag. In a collaborative study with Dr. Gerin, we are currently surveying HB Ag-positive plasma for units rich in Dane particles. When such units are found, attempts will be made to purify the Dane particle and isolate the internal

component, which will be used for the preparation of specific antibody for RNP. Evidence from several sources suggests that the internal component of the Dane particle may be the nucleocapsid of the hepatitis B virus.

(8) Transmission of Hepatitis B Virus to Sub-human Primates - In a collaborative study with Dr. William London (NINDS) sub-human primates of several species (rhesus monkey, African green monkey, Cebus monkey, Patas monkey, Wooly monkey, and Marmoset) have been inoculated with HB Ag-positive plasma in an attempt to transmit hepatitis B to these animals. Evidence for serial transmission of an agent associated with HB Ag in the rhesus monkey has been obtained; 5 serial passages (dilution factor of $10^{-15.5}$) have been completed and a pool of potentially infectious material prepared for further study. Infection of the rhesus monkey was documented by the detection of a transient HB Ag antigenemia following an incubation period of 8-13 weeks and the subsequent development of anti-HB Ag temporally related to the antigenemia. However, none of the animals developed clinical or sub-clinical hepatitis. Nevertheless, the rhesus monkey should prove a valuable tool for the characterization of the agent of hepatitis B. Such studies are currently in progress.

In a collaborative study with Dr. James Maynard (CDC, Phoenix, Arizona) chimpanzees have been inoculated with HB Ag-positive plasma in an attempt to transmit hepatitis B to a sub-human primate more closely related to man. Two chimpanzees so inoculated had evidence of infection, both animals developed anti-HB Ag and one of the animals demonstrated a transient HB Ag antigenemia just prior to seroconversion. However, neither animal developed clinical or sub-clinical hepatitis. Attempts to serially transmit the agent in the chimpanzee as well as attempts to determine the spectrum of clinical response to the agent in the chimpanzee are currently in progress.

As a part of this study, and in collaboration with Dr. Thomas Edgington (Scripps Institute), specimens of liver and other tissue from rhesus monkeys and chimpanzees infected with the agent of hepatitis B are being studied for immunofluorescent evidence of HB Ag in the tissues. These studies, in their initial stages, will include immunofluorescent staining for each of the sub-types of HB Ag, as well as attempts to identify and localize the internal core component of the Dane particle. If antigens are detected, the tissues will also be examined for evidence of binding of immunoglobulins to the infected cells. The results of such studies are expected to increase our knowledge of the immunopathology of hepatitis B infection.

(9) Cellular Immune Response to Hepatitis B - Attempts to determine the relative importance of cellular and humoral immune responses to hepatitis B in the clinical pathology of the disease have not been entirely successful, in part because the techniques of measuring cellular immune response are cumbersome and not highly reproducible. However, success with the lymphocyte transformation and macrophage migration inhibition techniques for the detection of cellular immune response to hepatitis B infection have been reported, but not confirmed. Recently, Dr. Feinstone (LID) has employed another technique for

detecting cellular immune response (release of radioactive chromium from sensitized red blood cells by convalescent lymphocytes) for the detection of prior exposure to HB Ag. Preliminary results with this technique, carried out in rhesus monkeys, appear very encouraging. This technique will be evaluated in a collaborative study of hepatitis patients, to be carried out with Dr. Hyman Zimmerman of the Washington, D.C. V.A. Hospital.

Significance to Biomedical Research and the Program of the Institute:

Hepatitis represents one of the major unsolved infectious disease problems. In addition, changing cultural conditions (the rise of the drug culture) has resulted in a rise in the relative importance of the more serious hepatitis B. Finally, the development of sensitive serologic techniques for the demonstration of prior exposure to hepatitis B has revealed that such infection is much more prevalent in the general population than was previously thought. Thus, hepatitis B is emerging as a public health problem of considerable magnitude. The present program is consistent with the institute's mission i.e., responsibility for the study of and, where practicable, the development of vaccines or other suitable methods for the control of infectious diseases. The recent rapid increase in our knowledge of hepatitis B has opened the way for improved control methods, including, possibly, active and passive immunization. The program of the hepatitis unit, LID, NIAID is directed primarily toward the realization of these goals.

Proposed Course of Project:

We plan to concentrate our studies of hepatitis B in the following areas: (a) expansion of our studies of hepatitis B in sub-human primates with the twin goals of characterizing the agent of hepatitis B and developing an animal model for the study of clinical aspects of hepatitis B infection, (b) improvement of serologic tests for HB Ag and their use in seroepidemiologic studies, (c) extension of biophysical studies of HB Ag and anti-HB Ag interactions, (d) attempts to grow the agent of hepatitis B in tissue and organ culture, and (e) through the use of seroepidemiologic and animal model studies, to better understand the parameters of active and passive immunization against hepatitis B. In addition, we plan to extend and modify the techniques developed with HB Ag to studies of HB Ag-negative hepatitis. These studies will include the modification of serologic techniques for use with hepatitis A as well as attempts to establish an animal model system for the study of hepatitis A.

Honors and Awards:

Invited participant Symposium on Viral Hepatitis and Blood Transfusion, San Francisco, California, March 25-26, 1972.

Publications:

Purcell, R.H., Holland, P.V., Wong, D.C., and Morris, J.A.: Viral hepatitis and hepatitis-associated antigen in the Far East. J. Infect. Dis. (In press, 1972).

Alter, H.J., Holland, P.V., and Purcell, R.H.: Counter Electrophoresis for Detection of Hepatitis-Associated Antigen: Methodology and Comparison with Gel Diffusion and Complement Fixation. J. Lab. Clin. Med. 77: 1000-1010, 1971.

Lander, J.J., Giles, J.P., Purcell, R.H., and Krugman, S.: Viral Hepatitis Type B (MS-2 strain): Detection of Antibody Following Primary Infection. New Eng. J. Med. 285: 303-307, 1971.

Gerin, J.L., Holland, P.V., and Purcell, R.H.: Australia Antigen: Large-scale Purification from Human Serum and Biochemical Studies of its Proteins. J. Virol. 7: 569-576, 1971

Lander, J.J., Holland, P.V., Alter, H.J., Chanock, R.M., and Purcell, R.H.: Antibody to Hepatitis-associated Antigen: Frequency and Pattern of Response as Detected by Radioimmunoprecipitation. J.A.M.A., In press, 1972.

Dietzman, D.E., Madden, D.L., Sever, J.L., Lander, J.L., and Purcell, R.H.: The Relationship Between Mongoloid Births and Maternal Exposure to Australia Antigen. Amer. J. Dis. Child., In press, 1972.

London, W.T., Lander, J.J., and Purcell, R.H.: Serial Transmission in Rhesus Monkeys of an Agent Associated with Australia Antigen (hepatitis-associated antigen). J. Infect. Dis., In press, 1972.

Maynard, J.E., Berquist, K.R., Krushak, D.H., and Purcell, R.H.: Experimental Infection of Chimpanzees with the Virus of Hepatitis B. Nature, In press, 1972.

Holland, P.V., Walter, H.J., Purcell, R.H., Walsh, J.H., Morrow, A.G., Schmidt, P.J.: Infectivity of Blood Containing the Australia Antigen. Proceedings of the Symposium on Australia Antigen. Amer. Soc. Microbiol., Phila., Pa., 1971. In press, 1972.

Alter, H.J., Holland, P.V., Purcell, R.H., Lander, J.J. and Feinstone, S.M.: Post-transfusion Hepatitis After Exclusion of Commercial and Australia-Antigen Positive Donors. Proceedings of Symposium on Australia Antigen, 1971. Amer. Soc. Microbiol. (In press, 1972).

Serial No. NIAID 70E
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biological and Biophysical Studies of Rhinoviruses

Previous Serial Number: Same

Principal Investigator: Dr. John C. Perkins

Other Investigator: Dr. Robert F. Buscho
Dr. Albert Z. Kapikian

Cooperating Units: None

Contract Support:

Contract Number	Project Off. (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589
Interagency Agreement	Dr. Chanock	Lorton Ref.		64,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:				
Professional:	8/12			8/12
Other:				

Project Description:

Objectives: (1) To develop and characterize temperature sensitive (ts) conditional lethal mutants of rhinoviruses for potential use in a live attenuated vaccine; (2) to study the local respiratory tract antibody response to rhinovirus antigens.

Methods Employed and Major Findings:

Evaluation of a 5-FU and Proflavine Induced ts Mutant of R1A Virus in Volunteers

In last year's annual report we reported that when $10^{4.7}$-$10^{5.0}$ $TCID_{50}$ of the most stable of the 5FU-induced ts mutants of R1A virus were administered to 14 seronegative volunteers intranasally, 9 of the 14 developed typical common cold-like illnesses indicating that the mutant did not possess the desired degree of attenuation. Virus isolates were analyzed for wild type revertants, and only 2 men, both of whom had not developed illness on challenge, were found to shed such virus. These findings indicated that reversion of the mutant to wild type was not responsible for the virulence of the ts mutant suspension administered to the volunteers.

In an attempt to develop a stable ts mutant which would produce little or no illness, the FU treated ts virus was grown in the presence of proflavine, another mutagen. A ts R1A pool (#1018) derived from R1A virus which had been treated with two separate chemical mutagens as noted above was prepared. After appropriate safety tests were performed, $10^{4.7}$ $TCID_{50}$ of this ts mutant was administered to 11 volunteers (10 of whom had prechallenge serum antibody titers ranging from <1:4 to 1:8; one had a titer of >1:8. Nasal antibody titers ranged from <1:2 to 1:8). Five of the 11 volunteers developed a common cold-like illness following challenge. Virus was recovered from 10 of the 11 men; certain isolates were selected for typing and all were typed as R1A. Virus isolates were also analyzed for evidence of wild type revertants and none was found, indicating that reversion of the mutant ts strain to wild type was not responsible for the virulence of the ts mutant suspension. It was of interest that in the small numbers studied the occurrence of illness did not appear to correlate with the presence or absence of either serum or nasal antibody in prechallenge specimens.

Significance to Biomedical Research and the Program of the Institute:

The use of conditional lethal mutants represents a promising approach to the development of live attenuated vaccines, although up to the present time such rhinovirus mutants have not possessed the desired degree of attenuation. In addition, studies of adenovirus mutants may help to explain the extreme antigenic lability of the rhinoviruses which now comprise at least 90 distinct serotypes.

Proposed Course of Project:

As noted above, the mutants tested thus far have not possessed the desired degree of attenuation. When additional methods for attenuating these viruses are available, further attempts to attenuate them will be made.

Honors and Awards: None

Publications: None

Serial No. NIAID 72A
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: New Techniques for Recovery of Respiratory Tract Viruses

Previous Serial Number: Same

Principal Investigator: Dr. Albert Z. Kapikian

Other Investigator: Dr. Robert M. Chanock

Cooperating Units: Mr. W. Lee Cline, Serology Unit, LID, NIAID

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (Part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	14/12	<1/12		14/12
Professional:	11/12	<1/12		11/12
Other:	3/12	<1/12		3/12

Project Description:

Objectives: (1) To recover new viruses from patients with respiratory disease; (2) to characterize such agents and study their biologic and biophysical properties; (3) to develop new techniques for the detection of respiratory tract viruses.

Methods Employed:

Specimens obtained from patients in the NIH, LID Common Cold Study have been studied intensively for the presence of viruses by both conventional and new techniques. The specimens are first examined by routine cell culture methods, and only those which fail to yield viruses are studied further in experimental culture systems--such systems include new cell cultures and human embryonic tracheal (HET) organ cultures. The use of new culture systems has been especially fruitful in the recovery of virus strains belonging to the human coronavirus group of viruses.

Fifteen of the 24 described coronavirus strains were first recovered by this laboratory employing new culture systems. However, study of the coronaviruses has been hampered by the lack of convenient and suitable methods for their

propagation. Nine of the 24 coronavirus strains were originally isolated in HET organ cultures only, while the remaining 15, all of which were similar to a prototype strain designated 229E, were isolated in monolayer tissue cultures. Since the coronaviruses appear to be important etiologic agents of adult upper respiratory tract illnesses continued intensive efforts have been made to develop new ways to detect these agents. Progress in the detection of coronaviruses is outlined in the following sections; in addition, the recovery of rhinoviruses by the use of new techniques is also presented.

Major Findings:

Detection of a New Coronavirus Strain by Immune Electron Microscopy - A nasal washing obtained from a 29 year old male (volunteer 692) in the NIH, LID Common Cold Study was inoculated into cell cultures of MK, HEK, HDCS WI38, HEP2, and later into human embryonic intestine (HEI) MA177 when this cell culture became available; no cytopathic agent was detectable following 2 passages in HEK and one passage in the remainder of these cell cultures. The nasal washing was carried through 3 passages in HET OC and the harvests of the third passage were examined for the presence of virus particles utilizing the technique of "immune electron microscopy". This technique was employed so that virus particles would appear in the form of "aggregates" rather than indistinct individual units thereby facilitating the visualization of low titered preparations. In this procedure 0.1 ml of the HET OC harvest was incubated at room temperature for 1 hour with 0.1 ml of uninactivated convalescent serum from patient 692; 0.8 ml of PBS was then added and the mixture was centrifuged at 17,000 rpm for 1-1/2 hours; the supernate was discarded and the pellet resuspended with a few drops of distilled water, stained with 3% PTA, and placed on a formvar-carbon coated grid; the excess fluid was removed with the edge of a filter paper disc and the grid examined under the electron microscope at a magnification of 40,000. A control preparation in which PBS was used instead of serum was treated similarly. Electron microscopic examination revealed the presence of numerous coronavirus aggregates with an abundance of antibody molecules coating the projections of most particles; in a few instances there was a suggestion of "holes" in the viral envelope, a probable result of the action of complement in conjunction with specific antibody. Examination of the virus-PBS mixture which had been treated exactly as above revealed no definite virus particles. Studies were performed to determine if strain 692 was related antigenically to coronaviruses 229E and OC43. A neutralization test was performed by "immune electron microscopy" using the presence or absence of aggregation as the measure of neutralization. In this test, strain 692 was incubated as described above with (1) a 1:20 dilution of the convalescent serum of patient 692, (2) a 1:20 dilution of 229E guinea pig antiserum, (3) a 1:20 dilution of OC43 mouse serum lot 1571, and (4) PBS; preparatory procedures for electron microscopic examination were as described above. Viral aggregates were observed in the virus suspension incubated with the convalescent serum, whereas no definite virus particles were observed in any of the other preparations, indicating that by this test system strain 692 was distinct from coronaviruses 229E and OC43. Further studies were done to determine the pathogenicity of virus 692 for suckling mice since OC43 virus induces encephalitis in suckling mice, whereas 229E virus does not. Strain

692 was inoculated IC and IP into two litters of NIH general purpose mice; after 19 days the mice showed no signs of illness and were sacrificed and stored at -70°C. Subsequently 20% mouse brain harvests were inoculated into two additional litters of suckling mice and after 26 days no illness was observed. Serologic studies of the patient's paired sera were difficult to interpret and inconclusive since the acute serum had been pooled with several other sera by error. Cell culture neutralization tests revealed that the convalescent serum titered 1:8 vs strain 229E (acute was 1:8) and 1:512 vs OC43 (acute was 1:128). Such titers are consistent with other epidemiologic studies in which neutralizing antibody titers to OC43 virus were at consistently higher levels than those of 229E virus. Attempts to adapt the HET OC harvests into various cell culture systems have thus far not been successful. It is not possible at this time to ascertain whether strain 692 is distinct from OC strains 16, 37, 44, 48 (all of which have not been able to be propagated in any known cell culture system) and B814 (which has not been able to be grown in this laboratory). The antigenic relationships among these fastidious agents awaits the discovery of a suitable system for their propagation.

Recovery of Presumptive Coronavirus Strains by Tissue Culture Techniques - We have continued our efforts to recover coronaviruses by cell culture techniques. In a previous annual report we described the recovery of 9 "229E-like" viruses, 8 of which were isolated directly in HEI MA177 cell cultures only. The HEI MA177 cell culture system continues to be the most sensitive cell culture available for the recovery of coronaviruses. Utilizing such cultures we have been able to recover 6 additional "229E-like" viruses and 1 presumptive coronavirus strain from patients enrolled in the NIH, LID Common Cold Study. A brief description of these strains follows:

(1) Strain 886 was recovered from a nasal washing obtained from a 29 year old male. Inoculation of the nasal washing into conventional cell cultures did not yield any cytopathic agent; however, subsequent inoculation of the nasal washings into HEI MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile and had characteristic coronavirus morphology on examination by both "immune electron microscopy" and conventional electron microscopic techniques. It was typed as 229E virus in a neutralization test with 229E guinea pig antiserum. Acute and convalescent sera from patient 886 were tested by the CF technique with a battery of respiratory viral antigens, and M. pneumoniae, and a significant rise in CF antibody to 229E virus was detected.

(2) Strain 884 was recovered from a nasal washing obtained from a 21 year old female. Inoculation of the nasal washing into conventional cell cultures did not yield any cytopathic agent, However, subsequent inoculation of the nasal washing into HEI-MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile; this agent was typed as 229E in a neutralization test with 229E guinea pig antiserum. Acute and convalescent sera were tested by the CF technique as above and demonstrated a significant increase in antibody to 229E virus.

(3) Strain 1133 was recovered from a nasal washing obtained from a 51 year old male. Inoculation of the nasal washing into conventional cell cultures did not yield any cytopathic agent on initial passage. However, after blind passage in HEK and subsequent initial passage of the nasal washing into MA177 cell cultures a cytopathic agent was recovered; the MA177 harvest was studied further and found to be acid and chloroform labile and to have typical coronavirus morphology by "immune electron microscopy" and by conventional electron microscopic techniques. This virus was also subsequently typed as 229E in a neutralization test with virus 229E guinea pig antiserum. The patient's acute and convalescent sera were tested by the CF technique as above and no increase in antibody was found. Additional testing will be carried out in an attempt to further characterize this agent.

(4) Strain 1142 was recovered from a nasal washing obtained from a 49 year old male. Inoculation of the nasal washing into conventional cell cultures did not yield any cytopathic agent on initial passage; however, blind passage into HEK cell culture and initial passage of the nasal washing into MA177 cell cultures yielded a cytopathic agent; the MA177 cell culture harvest was studied further and was found to be acid and chloroform labile and to have typical coronavirus morphology by "immune electron microscopy" and by conventional electron microscopic techniques. This virus was subsequently typed as 229E in a neutralization test with virus 229E guinea pig antiserum. Acute and convalescent sera were tested by the CF technique as above and no significant increase in antibody was found. Additional testing will be performed in an attempt to further characterize this agent.

(5) Strain 1152 was recovered from a nasal washing obtained from a 50 year old male. Inoculation of the nasal washing into conventional cell cultures yielded no cytopathic agent on initial passage; however, blind passage in HEK cell culture and initial passage of the nasal washing into MA177 cell cultures yielded a cytopathic agent which was acid and chloroform labile. This virus was subsequently typed as 229E in a neutralization test with virus 229E guinea pig antiserum. Acute and convalescent sera tested by the CF technique as above demonstrated a significant increase in 229E antibody. Additional testing will be performed to further characterize this agent.

(6) Strain 878 was recovered from a nasal washing obtained from a 57 year old made. A nasal washing inoculated into conventional cell cultures yielded no cytopathic agent; however, a nasal washing inoculated into MA177 cell cultures yielded a cytopathic agent which was acid and chloroform labile. This virus was subsequently typed as 229E in a neutralization test with 229E virus guinea pig antiserum. Acute and convalescent sera were tested by the CF technique as above and a significant increase in 229E virus antibody was found. This agent is being further characterized.

(7) Strain 859 was recovered from a nasal washing obtained from a 30 year old male. Inoculation of the nasal washing into conventional cell cultures yielded no cytopathic agent on initial passage; however, blind passage in HEK cell culture and initial passage of the nasal washing into MA177 cell cultures yielded a cytopathic agent which was found to be acid and chloroform labile.

Attempts to visualize the agent by immune elctron microscopy, using the patient's convalescent serum, 229E guinea pig serum, and OC43 mouse serum have not been successful. The agent has not been neutralized by either virus 229E guinea pig or virus OC43 mouse antisera in conventional cell culture neutralization tests. Acute and convalescent sera from patient 859 were tested by the CF technique as above and no significant increase in antibody was found. Additional testing will be performed to further characterize this agent.

Utilization of HET OC for Recovery of Rhinoviruses - Nasal washings obtained from patients enrolled in the NIH, LID Common Cold Study are routinely inoculated into MK, HEK, HEP2, HEI MA177 cell and HDCS WI38 cultures; a blind passage is routinely performed in the HEK cell cultures. If no cytopathic agent is detected in such cultures, the nasal washing is routinely passaged 3 times in HET OC. The harvest from the third organ culture passage is inoculated into the cell cultures mentioned above and if no CPE is observed, the HET OC harvest is examined under the electron microscope by both "immune electron microscopy" and conventional electron microscopic techniques. The four presumptive rhinoviruses outlined below were recovered in cell cultures inoculated with HET organ culture harvests:

(1) Strain 812 was recovered from a nasal washing obtained from a 32 year old male in HEI MA177 and HDCS WI38 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable; it has not been neutralized by sera containing at least 20 antibody units to each of the 90 numbered rhinoviruses. Further characterization is in progress.

(2) Strain 674 was recovered from a nasal washing obtained from a 21 year old female in HEI MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable. This nasal washing had originally induced a CPE in human aorta (A-39) cell cultures but the harvest was negative on subsequent passage.

(3) Strain 746 was recovered from a nasal washing obtained from a 19 year old female in HET MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable and has been typed as Rhinovirus 75.

(4) Strain 848 was recovered from a nasal washing obtained from a 23 year old female in both HDCS WI38 and HEI MA177 cell cultures following 3 HET OC passages; it produced a "rhinovirus-like" CPE and was acid labile and chloroform stable. Further study is in progress.

Significance to Biomedical Research and the Program of the Institute:

The search for new agents using the techniques outlined above is of importance in identifying possible etiologic agents in respiratory disease and as an initial step toward the ultimate goals of prevention and therapy. The discovery of new agents such as those described above attests to the value of this endeavor.

Further study of the biologic and biophysical nature of these viruses,and the epidemiology and pathogenesis of diseases associated with them can be expected to bring us closer to these goals.

Proposed Course of Project:

The search will continue for new agents from patients with respiratory disease using the techniques outlined in this report, and any new systems which appear to be profitable. In addition, further attempts will be made to adapt the organ culture strains to cell culture and to devise new methods for their study.

Honors and Awards:

Invited member of the World Health Organization, Food and Agriculture Organization of the United Nations Working Team on Coronaviruses in the WHO/FAO Program on Comparative Virology.

Invited participant at NIAID International Workshop on Coronaviruses, NIH, Bethesda, Maryland, Jan 31, 1972.

Delivered paper "Clinical, Epidemiologic and Laboratory Considerations of Coronaviruses of Human Origin" at NIAID International Workshop on Coronaviruses, NIH, Bethesda, Maryland, Jan. 31, 1972.

Invited participant at meeting of members of WHO/FAO Working Team on Coronaviruses, Feb. 1, 1972, NIH, Bethesda, Maryland.

Invited member of International Coronavirus Study Group.

Member NIAID Clinical Research Committee

Publicatcns:

Bucknall, R.A., Kalica, A.R., and Chanock, R.M.: Intracellular Development and Mechanism of Hemadsorption of a Human Coronavirus-OC43. Proc. Soc. Exp. Biol. Med. 139: 811-817, 1972.

Bucknall, R.A., King, L.M., Kapikian, A.Z., and Chanock, R.M.: Studies with Human Coronaviruses. II Some Properties of Strains 229E and OC43. Proc. Soc. Exp. Biol. Med. 139: 722-727, 1972.

Kapikian, A.Z., James, H.D., Jr., Kelly, S.J., King, L.M., Vaughn, A.L., and Chanock, R.M.: Hemadsorption by Coronavirus Strain OC43. Proc. Soc. Exp. Biol. Med. 139: 179-186, 1972.

Serial No. NIAID 72B
1. Infectious Diseases
2. Respiratory Viruses and Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Respiratory Viruses and Mycoplasmas in Human Volunteers

Previous Serial Number: Same

Principal Investigators: Dr. Robert F. Buscho
Dr. Elias G. Chalhub
Dr. Brian R. Murphy
Dr. Robert M. Chanock

Cooperating Units: Dr. Louis Potash, Flow Laboratories
Dr. Richard Hornick, and Dr. Y. Togo, University of Maryland School of Medicine
Dr. Robert H. Parrott, and Dr. H.W. Kim, Children's Hospital of D.C.

Contract Support:

Contract Number	Proj. Officer (NIAID)	Contractor	Contractor's Proj. Dir.	Annual Funding Level
PH 43-68-1309 (part B)	Dr. Chanock	Flow Labs	Dr. Gilden	$ 177,589
PH 43-66-63	Dr. Chanock	Univ. of Md.	Dr. Hornick	56,546
Interagency Agreement	Dr. Chanock	Lorton Ref.		64,000

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	81/12			81/12
Professional:	45/12			45/12
Other:	36/12			36/12

Project Description:

Objectives: (1) To evaluate temperature sensitive mutants and recombinants of influenza A virus for evidence of attenuation in adult volunteers; (2) to determine if antibody against the neuraminidase enzyme of influenza A virus protects man against disease produced by this virus; (3) to characterize the serum and local respiratory tract antineuraminidase antibody response of man

to infection with wild type or attenuated influenza A virus; (4) to determine whether the attenuated ts recombinant of influenza A virus stimulates the production of interferon; (5) to evaluate the existence of immunologic memory in local respiratory tract antibody response to rhinovirus antigens.

Methods Employed:

(1) Influenza Virus - The subjects for these studies were volunteers from the District of Columbia Reformatory in Lorton, Virginia. The influenza virus suspensions used were subjected to extensive safety tests and adventitious agents were not detected. Men were selected on the basis of general good health and absence or low level of serum neutralizing antibody titer for influenza A (H3N2). Informed consent was obtained prior to participation in the study. The men were maintained in isolation in a dormitory at Lorton Reformatory for 2 weeks. Volunteers were examined daily by two physicians, and cultures, respiratory secretions, and blood samples were taken when appropriate. The men were inoculated intranasally with $10^{5.0}$ $TCID_{50}$ with one-half of the inoculum administered as a coarse droplet aerosol (DeVilbiss atomizer), while the remainder was pipetted slowly into the nares. Nasopharyngeal washings for virus isolation were collected in veal infusion broth daily and inoculated into rhesus monkey kidney roller tube cultures. Cultures were examined for presence of a hemadsorbing virus every 5 days and discarded after 20 days if negative. Virus isolates were identified by the hemadsorption-inhibition test. Serum antibody and nasal antibody levels were determined by the neutralization technique.

(2) Rhinovirus 13 - A group of 47 volunteers were vaccinated intranasally with 1 ml of inactivated rhinovirus 13 (RV13) vaccine on alternate days. The volunteers were divided into three groups based upon their level of pre-existing nasal RV13 neutralizing antibody. The serum and nasal wash neutralizing antibody responses of these volunteers were followed after vaccination and compared.

Major Findings:

(A) Temperature Sensitive Recombinants of Influenza Virus - As described before, we produced conditional lethal temperature sensitive (ts) mutants of influenza A/1965 (H2N2) virus with the aim of evaluating their attenuation for man and suitability for use in a live virus vaccine. Ts mutation offers the possibility of site-specific attenuation for the lower respiratory tract. Mutants which are markedly restricted in growth at 37°-38°C should probably be limited in replication in the lower respiratory tract, the major site of significant pathology, which has a temperature of 37°C. However, growth of the mutants should occur with reasonable efficiency in the cooler passages of the upper respiratory tract, which have a temperature of 32°-34°C. In this manner ts mutants could primarily grow in the upper respiratory tract and stimulate immunological defense mechanisms without producing symptoms in the lower tract. Ts mutants are often partially defective at permissive temperature

(32°-34°C) and this property offers the possibility of attenuation for the upper respiratory tract as well.

Previously 2 ts mutants of influenza A/1965 (H2N2) were produced by growing virus in the presence of 5-fluorouracil (5-FU). One mutant, ts-1, failed to produce plaques at 37°C or above, while the other mutant, ts-2, had an in vitro shutoff temperature of 39°C. In contrast wild type virus produced plaques in monolayer cultures with high efficiency at 40°C. Both mutants were genetically stable in vitro and both were restricted in their growth in the hamster's lower respiratory tract; ts-1 was more restricted in vivo than ts-2. Although both mutants grew poorly in the lungs they induced significant resistance in hamsters to subsequent challenge with wild type virus. Finally, the mutants were found to possess distinct genetic lesions.

Encouraged by the behavior of the ts-1 and ts-2 mutants in the laboratory we sought to evaluate their level of attenuation in man. Efforts to study this property of the mutants were frustrated by our failure to find volunteers who lacked serum antibody for these viruses. This was not surprising since the influenza A2 viruses had been epidemic on a number of occasions during the 1956-1968 interval. In clinical trials we found that the mutants did not produce disease in men selected for having the lowest possible serum antibody titers; in no instance, however, was the level of serum antibody less than 1:32. These findings did not mean that the mutants were attenuated since the wild type virus, similarly did not produce disease in comparable volunteers. The mutants, however, were infectious since 50% of the inoculated volunteers developed a serological response.

In order to approach the question of the relationship of virulence and ts mutation we produced recombinant viruses with the surface hemagglutinin (H3) of Hong Kong influenza virus and a ts lesion (or lesions) of the influenza A/1965 (H2N2) ts-1 or ts-2 mutant. This was done since a significant proportion of the adult population in our locality lacked serum neutralizing antibody for the H3 antigen when the studies to be described were performed.

A series of recombinants were produced during mixed infection of primary bovine kidney (BK) cells with wild type influenza A/Hong Kong/1968 (H3N2) virus and influenza A/Great Lakes/1965 (H2N2) ts-1 or ts-2 mutant. Recombinants were sought by plating the progeny from the mixed infection onto BK cell monolayers in the presence of H2 antiserum and characterizing the antigenic structure and ts phenotype of the resulting plaques. The genetically stable recombinants which were selected possessed the surface H3 antigen of the Hong Kong virus and a ts lesion (or lesions) of the 1965 influenza A virus. Recombinants derived from the ts-2 mutant had the same temperature sensitivity (i.e., shutoff temperature* of 39°C) as the parent mutant. Further, these recombinants did not complement each other in pair-wise infection suggesting that they shared a common ts lesion acquired from the ts-2 mutant. In the current study the influenza A (H3N2)-ts-2[C] recombinant was evaluated in volunteers.

*Shutoff temperature refers to the lowest temperature at which a 100-fold decrease in efficiency of plaque formation was observed.

Recombinants derived from the ts-1 mutant exhibited 3 different patterns of temperature sensitivity. Like the parent ts-1 mutant, one class of recombinants had a shutoff temperature of 37°C, while recombinants belonging to the other two classes had restrictive temperatures of 38°C and 39°C, respectively. This suggested that the ts-1 mutant contained 3 discrete ts lesions and that these were segregated during recombination with the influenza A/Hong Kong/1968 (H3N2) virus. This suspicion was confirmed by complementation analysis which showed that the 37°C, 38°C and 39°C ts-1 recombinants possessed distinct genetic lesions. In this study the influenza A (H3N2) ts-1[A] recombinant (shutoff temperature of 37°C) and the influenza A (H3N2) ts-1 [E] recombinant (shutoff temperature of 38°C) were evaluated in volunteers.

Before the 3 recombinants were administered to volunteers it was ascertained that these viruses were moderately or markedly restricted in their growth in the hamsters' lungs. Furthermore, infection of hamsters with the recombinants induced significant resistance to later challenge with wild type influenza A/ Hong Kong/1968 (H3N2) virus.

A mutant to be used for immunoprophylaxis of influenzal disease must strike a fine balance with respect to attenuation on the one hand and capacity to stimulate the hosts' protective mechanisms on the other. Two of the recombinants, (ts-1[A] and ts-2[C]),failed to achieve this desired balance. The ts-1[A] recombinant was too attenuated to infect seronegative volunteers, while the ts-2 [C] recombinant appeared to be insufficiently attenuated and was thus not acceptable for purposes of immunoprophylaxis.

Fortunately the 3rd recombinant, ts-1[E], appeared to possess the desired degree of attenuation. The virus was capable of infecting almost every seronegative volunteer given 10^6 $TCID_{50}$ by the nasopharyngeal route. However, this virus was sufficiently defective so that the majority of infected individuals did not develop symptoms. Those symptoms which occurred were mild and were considered acceptable when balanced against the disease to be prevented. Infection with the recombinant was extensive enough, however, to stimulate a moderately high titer of serum and nasal wash neutralizing antibody. In view of this immunologic response it was not surprising that volunteers infected with the ts-1[E] recombinant were completely resistant to influenzal disease produced by a virulent wild type virus challenge. The ts-1[E] recombinant possessed 2 other properties desirable in a vaccine strain. The recombinant appeared to be genetically stable during replication in the respiratory tract of man and infection did not spread from infected individuals to susceptible cohorts despite close contact during a time when the former group shed virus.

In the initial clinical studies the virus given to volunteers was grown in primary bovine kidney tissue culture. This was the culture system in which the genetic manipulations were performed which led to the development of the recombinants. When it became clear that the ts-1[E] recombinant was a promising vaccine candidate another host system was sought which would meet current requirements for a licensed biological product. For this reason the ts-1[E]

virus was grown in avian leukosis virus free eggs, a substrate already licensed for the production of human vaccines. 10^6 $TCID_{50}$ of egg-grown virus was given to 8 seronegative men by the intranasal route. Seven volunteers shed virus but only one of the men developed mild coryza not associated with any other sign or symptom. This response resembled that seen following administration of a comparable quantity of bovine kidney culture grown virus.

B. Role of Antibody Against Influenza A Neuraminidase in Resistance to Infection with Influenza A Virus - Influenza A virus has two structurally, functionally, and genetically distinct glycoproteins on its surface--the hemagglutinin and neuraminidase. In man, resistance to infection with influenza A virus has usually been assessed by measuring hemagglutination-inhibiting and neutralizing antibodies--both of these antibodies being induced by the surface hemagglutinin. There is substantial evidence that anti-hemagglutinin antibody confers resistance to influenzal illness in man. In contrast, there is no information concerning the role that anti-neuraminidase antibody (ANAB) may play in resistance to human influenza although studies in other species have indicated that it may also have a protective effect. The appearnce of the Hong Kong subtype of influenza A in 1968 provided us with an opportunity to study the role of ANAB, independent of anti-hemagglutinin antibody, in resistance of man to influenza A virus. This new subtype has a hemagglutinin immunologically distinct from the influenza A virus subtype that circulated from 1957 to 1968, but these two subtypes have related neuraminidase antigens. This change in hemagglutinin antigen permitted us to evaluate the response to the Hong Kong influenza A virus of men who lacked antibody to the hemagglutinin but who had varying levels of ANAB as a result of natural infection with the preceding influenza A virus subtype. Volunteers lacking serum or nasal wash neutralizing antibody to H3 hemagglutinin, but possessing varying levels of serum ANAB, were inoculated intranasally with 10^5 $TCID_{50}$ of influenza A/Hong Kong/1968 (H3N2) virus. The severity of the clinical response correlated with the level of serum ANAB at the time of virus challenge. The group of 7 volunteers who failed to develop illness had the highest mean level of serum ANAB. Despite their failure to develop illness these volunteers each became infected as manifested by virus isolation and/or a four-fold rise in serum or nasal wash antibody. Another group of 8 volunteers who developed febrile influenzal illness had the lowest mean level of serum (ANAB). These 8 volunteers shed a larger amount of virus for a longer duration than men in the first group. Six volunteers who developed afebrile illness had levels of (ANAB) intermediate between these two groups. The role that ANAB present in local respiratory tract secretions might play in resistance could not be evaluated since this antibody was not detected in any of the nasal secretions of 18 of the 21 men from whom nasal washes were available.

The findings from this study suggested that ANAB provided protection against illness experimentally induced by influenza A (H3N2) virus and are analagous to the experiments of Shulman and associates who studied influenza A (H2N2) infection in mice. Our findings suggest that efforts should be made to stimulate ANAB as well as anti-hemagglutinin antibody for more effective prophylaxis of influenzal disease.

C. Nasal Wash and Serum Anti-neuraminidase Response to a Wild Type and Attenuated Hong Kong Influenza Virus - In man, antibody to the neuraminidase antigen on the surface of the influenza A virion is associated with resistance to influenza A virus. It was suggested that more effective prophylaxis of influenzal disease might be achieved by stimulating antibodies to the neuraminidase as well as the hemagglutinin. It was, therefore, of interest (1) to evaluate the serum and nasal wash anti-neuraminidase antibody response of volunteers to our live attenuated temperature sensitive recombinant virus, influenza A (H3N2) - ts-1 [E]; (2) to compare the serum and nasal wash response to this recombinant virus with that of the wild type virus; and (3) to determine the relative contribution of antibody to the neuraminidase and neutralizing antibody to resistance to influenza A virus infection in man. Seven volunteers received $10^{4.5}$ $TCID_{50}$ of wild type influenza A/Hong Kong/68 (H3N2) and 17 volunteers received $10^{6.0}$ $TCID_{50}$ of influenza A (H3N2) - ts-1[E]. These volunteers were seronegative with respect to the hemagglutinin but possessed varying levels of antibody to the neuraminidase. All men presumably had immunological experience with the neuraminidase antigen as a result exposures to the influenza A viruses (H2N2) that circulated between 1957-1967 so that the immunological response that we measured was a primary immunological response to hemagglutinin (H3) but a secondary response to the neuraminidase (N2). Seven of the seventeen men who received the attenuated live ts virus vaccine developed a two-fold or greater rise in serum ANAB with a rise in geometric mean titer from 1:2.8 prior to infection to 1:5.0 on day 14. Six of the seventeen men developed a nasal wash anti-neuraminidase response. A total of 8 of the 17 men had a rise in serum or nasal wash anti-neuraminidase antibody. However, by day 28, 10 of the 17 men had levels of serum anti-neuraminidase that were earlier associated with resistance to influenza A virus (H3N2). In contrast to the volunteers who received the ts recombinant virus, each of the 7 volunteers who received wild type virus (H3N2) had a two-fold or greater rise in serum anti-neuraminidase with a rise in a geometric mean titer from 1:2.5 prior to infection to 1:38 on day 14. Six of the seven volunteers developed anti-neuraminidase antibody in their nasal secretions. The other man had antibody in nasal secretions prior to infection. These results indicated that the live attenuated ts recombinant virus stimulated low levels of ANAB in less than 50% of the men in contrast to wild type virus which stimulated high levels of ANAB in 100% of the volunteers.

Twelve of the men who had received the ts recombinant virus were available for wild type virus challenge 35 days after receiving the recombinant virus. One group of five men lacked serum or nasal wash ANAB, but each possessed nasal wash neutralizing antibody (geom. mean titer 1:33) and 3 of the 5 possessed serum neutralizing antibody. Another group of seven volunteers possessed serum or nasal wash ANAB and, in addition, serum and nasal wash (geom. mean 1:34) neutralizing antibody. None of the volunteers in either group developed illness or shed virus. Thus, men who lacked serum or nasal wash ANAB but who possessed serum and nasal wash neutralizing antibody were protected from illness and failed to shed virus. This finding suggests that neutralizing antibody alone (or some factor associated with neutralizing antibody) provides resistance to reinfection with influenza A virus. The implications of these results are important for the future development of live

attenuated ts recombinant viruses. A hypothetical example will illustrate these points. The prevalent strain of influenza A virus for which a ts recombinant virus is available is designated H3N2-ts. The next new strain which emerges may have major antigenic changes in both surface antigens, H4N3, as occurred in 1957 (H1N1 → H2N2). If recombinant attenuated viruses are to be produced that possess the hemagglutinin of the new subtype (H4) and the ts defect, one must decide whether one should employ either of the following recombinations; H4N2-ts or H4N3-ts, as the new vaccine strain. The present results suggest that satisfactory resistance to influenza A virus can be induced by the hemagglutinin antigen alone. The fact that the H2N2 ts recombinant virus induced ANAB in less than 50% of an immunologically primed group of volunteers suggests that including the N3 neuraminidase in a new vaccine would add little to the protective effect of the vaccine. However, including the old N2 neuraminidase in the new vaccine might be potentially helpful for it might provide a basis for further attenuation of the new vaccine without affecting its ability to stimulate a serological response to the new hemagglutinin.

D. Interferon Induction by Wild Type and ts Recombinant Influenza A Viruses - One theoretical advantage of a live attenuated influenza virus vaccine over an inactivated vaccine is the possibility that a live virus vaccine might stimulate local non-specific resistance to infection in addition to immunologically specific protection. This type of non-specific resistance would begin before the onset of immunologically specific resistance and thereby would extend the period of protection to include the first several days after vaccine administration. This additional protective effect of live virus would be particularly useful when the vaccine is introduced into a community in which wild type virus is widely seeded as would be the case during a pandemic caused by a new antigenic subtype of influenza A virus. It is likely that some component of this non-specific resistance might be mediated by interferon. For this reason we studied the local nasopharyngeal interferon response in volunteers experimentally inoculated with wild type virus or a recombinant ts virus that was shown to possess many properties suitable for use as a vaccine strain. Volunteers lacking serum or nasal wash neutralizing antibody to the H3 hemagglutinin received either $10^{4.5}$ $TCID_{50}$ of influenza A/Hong Kong/68 (H3N2) wild type or $10^{6.0}$ of influenza A (H3N2)-ts-1[E] recombinant virus. Nasopharyngeal washes collected in tryptase phosphate broth were mixed with antibody to the Hong Kong virus and assayed on human embryonic kidney for interferon activity by a yield reduction method using Sindbis virus. Wild type virus stimulated interferon production in 6 of 7 men with a peak geometric mean titer of 1:41 on day 3 and interferon was detectable from day 2 through 7. The range of titers in the men in whom interferon activity was detected was 1:8-1:6000. Interferon activity was detected in 8 of 17 men receiving the recombinant virus with a peak geometric mean titer of 1:7 on day 2. This activity was detected from day 2 to day 5 with a range of 1:8 to 1:11. Interferon activity was detectable in the nasopharyngeal washes of approximately 50% of men receiving the ts recombinant virus. Because of the dilution factor involved in the nasopharyngeal wash, it seems reasonable to suggest that interferon might have been produced in more than 50% of the men. It is clear that the recombinant virus, like the wild type virus, stimulates interferon production, but whether any non-specific protection is afforded by this or other factors remains to be demonstrated.

E. Study of the Nasal Secretory Antibody Response to Rhinovirus Vaccination - Last year we showed that intranasal vaccination with an inactivated rhinovirus antigen induced resistance to challenge with live virus which lasted for at least six months following vaccination. The local neutralizing antibody which appeared following vaccination seemed to be specific for rhinovirus 13 and such local antibody persisted for at least 330 days following vaccination. There was some evidence for immunologic memory in the local respiratory tract secretory immune response, however, this was not established beyond question. For this reason additional studies were performed.

In the current study intranasal administration of inactivated vaccine (X2) did not stimulate a serum or local secretory antibody response in men who lacked these 2 antibodies. However, this quantity of vaccine did stimulate local nasal antibody in men who possessed a low level of both antibodies prior to vaccination. Men with high levels of both antibodies did not respond to vaccination, probably because the high pre-existing antibody titers either dampened a response or obscured it. These findings are compatible with the view that local respiratory antibody system possesses some degree of immunologic memory.

Significance to Biomedical Research and the Program of the Institute:

With respiratory tract pathogens, such as RS virus, influenza A virus and Mycoplasma pneumoniae, temperature sensitive (ts) mutants have consistently exhibited a pattern of diminished infection in the hamster, a host in which each of these agents replicates. Furthermore, there is a correlation between degree of limitation of infection and the extent of the temperature sensitivity of the mutant. Mutants with the lowest restrictive temperature exhibit the most limited growth in the lower respiratory tract, the region in which disease manifestations are most severe. Temperature sensitivity, i.e., the inability of the mutant to replicate at 37°-39°C, does not necessarily pose an impediment to its capacity to infect and replicate in the upper respiratory passages (nasal turbinates, etc.) since the temperature in this region varies between 32° to 34°C. However, there is also a correlation between defectiveness (i.e., shutoff temperature) of a mutant and capacity to grow in the upper respiratory tract. In the present study of ts recombinants of influenza A/HK/68 (H3N2) virus we observed the correlation between shutoff temperature and attenuation just described when 3 genetically distinct ts viruses were given to seronegative volunteers. The ts-1[A] recombinant, which exhibited a shutoff temperature of 37°C, was too defective to infect volunteers or induce resistance to challenge with wild type virus. The ts-2[C] recombinant, with a shutoff temperature of 39°C, was the least attenuated of the 3 recombinants and retained sufficient virulence so that it could not be considered for use in a live vaccine. Finally, the intermediate recombinant, ts-1[E] which had a 38°C shut-off temperature, exhibited an intermediate degree of attenuation which was appropriate for a virus to be used in a live vaccine for prevention of influenzal disease.

It was of interest that a discrete genetic lesion in each of 3 separate cistrons of the influenza A virus genome was associated with attenuation for man. This suggests that virulence of influenza A virus for man is a complex property which is influenced by the expression of more than one gene function. Similar evidence for the polygenic nature of virulence comes from experiments in hamsters with 4 distinct ts recombinants (including the 3 evaluated in this study) and in mice with 14 ts mutants of the WSN strain of influenza A virus which belong to 5 distinct recombination groups. Taken together these observations offer some encouragement for the eventual development of an acceptable, effective live vaccine since polygenic control of virulence implies that influenza A virus should be vulnerable to attenuation through alteration of any or almost any of its genes.

If recombination is the basis for major antigenic shift of influenza A viruses it is ironic and perhaps appropriate that this mechanism may prove to be of major importance in bringing these viruses under control. In this study, genetic recombination was used to transfer ts lesions from a 1965 strain of virus (H2N2) into the 1968 Hong Kong virus (H3N2). Of course, this method of transferring attenuation to a new influenza virus requires that the ts lesions not affect that portion of the genome which codes for the hemagglutinin, the antigen which stimulates neutralizing antibody. Since we were fortunate in inducing mutations in the non-hemagglutinin portion of the genome, transfer of ts lesions to the Hong Kong virus could be achieved. Transfer of ts lesions from influenza A (H2N2) to the Hong Kong virus (H3N2) occurred with high frequency and was accomplished in a short period of time. The facility with which this manipulation was performed suggests that it should be possible to effect a similar transfer of an appropriate ts lesion at the time the next major antigenic variant emerges. This approach offers the possibility for rapid development of a live vaccine to be used for containment of pandemic disease. Since we now have a ts lesion (ts-1[E]) which specifies the appropriate balance of attenuation and immunogenicity required of a vaccine virus the prospects for future live recombinant virus vaccines are reasonably hopeful.

Several significant advances were made in our understanding of the factors involved in protection against influenzal disease. During the past year we obtained the first evidence that antibody to the neuraminidase enzyme is related to resistance to human influenzal illness. These findings suggest that efforts should be made to stimulate anti-neuraminidase antibody as well as anti-hemagglutinin antibody for more effective prophylaxis of influenza. It should be emphasized that the anti-hemagglutinin antibody is more protective and can provide impressive resistance in the absence of anti-neuraminidase antibody. However, for maximal protection it would be desirable to induce both types of antibody.

One theoretical advantage of a live vaccine over an inactivated vaccine is the possibility that the former might stimulate an initial non-specific resistance before specific immunological resistance develops. If this occurred, the period of protection afforded by a live vaccine could include the first few days after vaccine administration. It was of interest, therefore, to find that the ts-1[E] influenza recombinant induced the development of detectable interferon in 50%

of the experimentally infected vaccinees. This suggests that the ts-1[E] virus might induce non-specific resistance to influenza prior to the development of specific immunologic protection.

Previous studies with the RV13 inactivated vaccine given intranasally have shown that it effectively induced resistance to experimental rhinovirus illness, whereas when administered intramuscularly it was without effect. This experience indicated that local intranasal vaccination was the more effective method for prevention of rhinovirus respiratory tract illness. The current findings suggest that the local respiratory tract antibody system has some degree of immunologic memory. This type of local recall may prove to be very important in the eventual success of local immunization in the respiratory tract against rhinoviruses as well as other respiratory pathogens.

Proposed Course of Project:

Evaluation of the ts recombinants of influenza A/Hong Kong/1968 (H3N2) virus will continue. Previously the ts-1[A], ts-1[E], and ts-2[C] recombinants were evaluated in seronegative volunteers. We plan to test a member of the 4th group of recombinants, ts-1[H], in order to complete our study of the temperature sensitive influenza A viruses in man. This will allow us to correlate certain in vitro properties of the recombinants with their level of attenuation and immunogenicity in man.

The egg adapted ts-1[E] recombinant will be evaluated in a large group of seronegative volunteers in order to confirm its attenuation and immunogenicity. Volunteers recently infected with the ts-1[E] recombinant will be challenged with wild type parainfluenza 1 virus to determine whether the influenza A recombinant stimulates non-specific resistance mechanisms so as to extend the period of protection provided by live influenza vaccine to include the first 2 weeks after vaccination.

Honors and Awards: None

Publications:

Buscho, R.F., Perkins, J.C., Knopf, H.L.S., Kapikian, A.Z., and Chanock, R.M.: Further Characterization of the Local Respiratory Tract Antibody Response Induced by Intranasal Instillation of Inactivated Rhinovirus 13 Vaccine. J. Immunol. 108: 169-177, 1972.

Hill, D.A., Baron, S., Perkins, J.C., Worthington, M., VanKirk, J.E., Mills, J., Kapikian, A.Z., and Chanock, R.M.: Evaluation of an Interferon Inducer in Viral Respiratory Disease. J. Amer. Med. Ass. 219: 1179-1184, 1972.

Hill, D.A., Baron, S., Levy, H.B., Bellanti, J., Buckler, C.E., Cannellos, G., Carbone, P., Chanock, R.M., DeVita, V., Guggenheim, M.A., Homan, E., Kapikian, A.Z., Kirschstein, R.L., Mills, J., Perkins, J.C., VanKirk, J.E., and Worthington, M.: Clinical Studies of Induction of Interferon by Polyinosinic·polycytidylic Acid. In Pollard, M. (ed.): From Molecules to Man - Perspectives in Virology VII. New York, New York, Academic Press, Inc., 1971, pp. 198-222.

Mills, J., Chanock, R.M., VanKirk, J., Bellanti, J.A., and Perkins, J.C.: Biology of Antibodies to Infectious Agents in Respiratory Secretions. Proceedings of the Conference on "Biologic Aspects and Clinical Uses of Immunoglobulins," sponsored by the Ohio State University College of Medicine, Columbus, Ohio, March 30, 1969, pp. 74-106, 1970.

Mills, J., VanKirk, J.E., Wright, P.F., and Chanock, R.M.: Experimental Respiratory Syncytial Virus Infection of Adults. Possible Mechanisms of Resistance to Infection and Illness. J. Immunol. 107: 123-130, 1971

Murphy, B.R., Chalhub, E.G., Nusinoff, S.R., Chanock, R.M.: Temperature-sensitive Mutants of Influenza Virus. II. Attenuation of ts Recombinants for Man. J. Infect. Dis., In press, 1972.

Murphy, B.R., Kasel, J.A., and Chanock, R.M.: Association of Serum Anti-neuraminidase Antibody with Resistance to Influenza in Man. New Eng. J. Med., In press, 1972.

Wright, P.F., Mills, J., and Chanock, R.M.: Evaluation of a Respiratory Syncytial Virus Temperature-sensitive Mutant in Adult Volunteers. J. Infect. Dis. 124: 505-511, 1971.

Serial No. NIAID 72C
1. Infectious Diseases
2. Epidemiology
3. Bethesda, Maryland

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Role of Delayed Hypersensitivity in the Immune Response to Respiratory Viral Infections

Previous Serial Number: Same

Principal Investigator: Roger E. Wetherbee, M.D.

Other Investigators: Robert F. Buscho, M.D.
Brian R. Murphy, M.D.
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Cooperating Units: Dr. H.W. Kim, Virus Research Laboratory
Children's Hospital of D.C.

Man Years:	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	15/12			15/12
Professional:	12/12			12/12
Other:	3/12			3/12

Project Description:

Objectives: (1) To study a population of children for (a) frequency of delayed hypersensitivity (D.H.) to parainfluenza 1 and 3, respiratory syncytial virus and influenza A2; and (b) to determine the correlation between D.H. and the presence of serum antibody to these viruses in this population. (2) To characterize the appearance and role of D.H. in experimental parainfluenza and influenza A2 infections of laboratory animals. (3) To examine aspects of D.H. manifested in vitro in organ fragment culture. (4) To establish a set of suitable viral and nonviral antigens for inducing D.H. in vivo and testing the presence of D.H. in vitro.

Methods Employed:

(1) In vitro Assays for D.H. - Migration Inhibitory Factor (M.I.F.), an effector substance released by sensitized lymphocytes exposed to the sensitizing antigen which inhibits the normal migratory behavior of macrophages and monocytic cells, is the basis of the principal in vitro assay systems used in this project. A second response of lymphocytes in vitro to an antigen to which they have become sensitized is dedifferentiation, DNA synthesis and cell division. Normal lymphocytes will also respond similarly to phytohemagglutinin. A lymphocyte

transformation test which quantitatively measures the tritiated thymidine uptake of cultured lymphocytes was used as a second assay system.

A. Human direct M.I.F. assay. Surgically excised tonsils transported in cold Hanks' balanced salt solution to NIH from Children's Hospital, of D.C. serve as the source of cells used in a direct, single step M.I.F. assay analogous to the guinea pig peritoneal exudate cell model where the cell suspension contains both effector and target cells.

B. Human indirect M.I.F. assay. Aliquots of cell suspensions prepared from surgically excised tonsil specimens are incubated for three days with antigens to be tested. The incubation fluids are harvested, concentrated, and assayed for M.I.F. activity using normal guinea pig peritoneal exudate cells as the indicator cells.

C. Human lymphocyte transformation. A cell suspension prepared from human tonsilla tissue is incubated with varying concentrations of phytohemagglutinin for three days and the uptake of tritiated thymidine then quantitated in a scintillation counter.

D. Guinea pig direct M.I.F. assay. Peritoneal exudate cells, principally lymphocytes and macrophages, are elicited by the intraperitoneal injection of sterile mineral oil via a percutaneous catheter. These cells are harvested by peritoneal lavage with Hanks' balanced salt solution, again by percutaneous catheter. To assure a sufficient quantity of cells, the harvests from several animals are combined. Only inbred guinea pigs are used to prevent a mixed lymphocyte incompatibility reaction. These cells are used as indicator cells in the human indirect assay or alone in the direct guinea pig assay.

2. Assays for Cell Mediated Cytotoxicity. Sensitized lymphocytes may exhibit direct cytotoxicity in the presence of the sensitizing antigen. An attempt to evaluate this phenomenon with respiratory viruses was made using small fragments of guinea pig trachea cultured in vitro and infected with either influenza A2 or parainfluenza 3 virus. Both viruses grow well in the respiratory epithelium of this tissue and a considerable amount of virus is shed into the fluid medium. Peritoneal exudate cells from control and virus sensitized guinea pigs are added to these infected tracheal fragments for varying periods of time and the effects of the added lymphocytes assayed by alteration in the production of infectious virus in the culture and by the occurrence of degenerative changes in the respiratory epithelium as seen by electron microscopy or light microscopy.

3. Production of Viral Antigen - Antigens intended for in vitro testing for the presence of D.H. must be highly purified or controlled by uninfected culture material of purification is not feasible. A staisfactory compromise has been achieved by growing influenza A2 and parainfluenza 3 viruses in guinea pig tracheal organ culture using protein free medium. A pool of harvest fluids from identical but uninfected cultures served as the control.

Antigen preparations for parenteral immunization of animals must be free of components found in media used in the assay systems or reactivity to these components will occur in the assays. A chicken egg allantoic fluid pool of influenza A2 virus has been prepared and used for this purpose.

4. Serologic Screening Techniques - Routine tube neutralization, plaque reduction, HAI, and complement fixation tests are used for screening and quantitation of serum and nasal wash specimens.

5. Sources of Test Subjects and Tissues - The population of children being screened for parainfluenza, respiratory syncytial, and influenza A2 infections is drawn from private patients at Children's Hospital of D.C. undergoing routine tonsilectomy. The surgical specimens of tonsil tissue are transported to NIH in cold Hanks' balanced salt solution. Inbred strain 2 and strain 13 guinea pigs are supplied as weanling males from the NIH animal production section.

6. Quantitation of M.I.F. Data and Statistical Analysis - Generally two chambers are employed for each antigen or control tested in the M.I.F. assay and there are two of usually three cell-packed capillaries per chamber. The projection of the migratory cell fan is traced on paper using an EPOI LP·6 profile projector and the tracing cut out and weighed on a Mettler balance. The weights of these cut-outs are compared using the student t-test and the probability of identity determined from t distribution tables.

Major Findings:

1. Occurrence of D.H. in Tonsillectomized Children - A series of direct M.I.F. assays using human tonsil cells from 27 pediatric patients were made using 25 ugm/ml PPD and 10% concentrations of sucrose gradient purified formalin inactivated preparations of parainfluenza 1 and respiratory syncytial virus. After overcoming initial technical difficulties a series of assays from 14 children have been subjected to statistical evaluation. The patients ranged in age from two years to 11 years with a mean of 5.6 years. The tonsils from eight patients manifested no inhibition of migration to any of the three antigens tested. The tonsil from one patient demonstrated significant inhibition ($P < .05$) to all three antigens and those from the five remaining patients each demonstrated significant inhibition ($p < .05$) to one of the antigens.

	PPD	Para 1	RS
Number of patients with inhibition	2 (14%)	3 (21%)	3 (21%)
Patient's age	(6), 7	5, (6), 6	(6), 9, (NA)

Age in parenthesis denotes same patient. NA = not available.

The mean age of the patients manifesting D.H. was 6.6 years, a year greater than the mean age of the entire group. It is not clear whether this difference has any significance. Forty-three percent of the patients demonstrated D.H. by the direct M.I.F. assay to at least one of the antigens. Unfortunately, no serum or nasal wash specimens were available for any of these patients so a correlation between D.H. and humoral antibody was not possible.

Analysis of the data in this series revealed a variation in extent of cell migration from one cell filled capillary to the next, apparently unrelated to antigen. This tendency has made analysis of the data difficult and has lead to an attempt to generate an antigen dose response curve in the direct assay and a comparison between the direct and indirect assays.

Of three tonsils so examined, from a four year old, a five year old and a seven year old, using PPD at five and 25 ugm/ml and a guinea pig tracheal organ culture grown influenza A2 virus suspension at one and five percent, none showed significant inhibition to PPD but one (H.T.A. #28) showed significant inhibition to one percent influenza virus. In none of the three could a convincing dose response curve be constructed. The indirect M.I.F. assay was performed on two of the specimens, including #28. Again, no inhibition in the presence of PPD was seen and only in #28 was inhibition seen to influenza. Thus, there was a correlation between the two assay methods. However, the indirect assay yielded data of substantially greater precision. Whereas, in direct H.T.A. #28, the level of inhibition seen to one percent influenza had statistical significance at the two percent level, the inhibition seen in the indirect assay was significant with $p < .001$.

Human tonsillar cell suspension from several patients have been examined for lymphocyte transformation in response to phytohemagglutinin. After a three day incubation period these cells do show a characteristic dose related increase in tritiated thymidine uptake.

It is clear from this study that delayed hypersensitivity to viral antigens does exist in a significant proportion of children in the age group under study. The role played by this sensitivity in the immunopathology of clinical viral respiratory illness is unknown.

2. Occurrence of D.H. in Seronegative Inbred Guinea Pigs Infected with Parainfluenza 3 - Preliminary studies revealed that weanling inbred guinea pigs as supplied by NIH animal production were seronegative for parainfluenza 3 virus. After intranasal inoculation with live, unadapted virus, infection occurred and virus grew in the lungs and trachea for 7-9 days.

In the first study, inbred strain 13 guinea pigs were inoculated intranasally with approximately $10^{6.0}$ $TCID_{50}$ parainfluenza 3 virus. A control group received only culture medium. Nine days post-inoculation four animals in each group were injected by the intraperitoneal route with oil and 12 days post-inoculation, the animals were sacrificed and the peritoneal exudate cells harvested and pooled in control and experimental groups. Direct M.I.F. assays were performed using guinea pig tracheal organ culture grown parainfluenza 3 virus. The control cells

showed no inhibition to the virus but the experimental cells showed clear inhibition in the presence of virus and a dose related response was observed.

A second study was undertaken to correlate virus shedding, serum antibody levels and development of D.H. In addition, the question of whether the viral antigenic mass in the inoculum alone suffices to induce D.H. in the inoculated animals was approached by including a group of animals receiving an inoculum of ether inactivated virus. The inactivated inoculum was shown to have nearly the same HA titer as the live virus inoculum but no infectivity. At no time in this study did any control animal or recipient of inactivated virus shed infectious virus. All live virus inoculated animals shed infectious virus through day 5. Forty-six percent shed virus on day 7 but none shed virus thereafter.

D.H. could not be detected in infected animals one day after inoculation by direct M.I.F. assay of peritoneal exudate cells. On day 6 the infected animals showed clear inhibition of migration, which was a dose related response. On day 12, control animals and animals receiving inactivated virus failed to show any significant inhibition, while the infected animals showed significant inhibition of migration. Twenty five days after inoculation, a group of infected animals continued to demonstrate clear inhibition of migration.

It appears that infectious replicating virus is necessary for the induction of D.H. Organ culture medium or inactivated virus had no effect when inoculated intranasally. Further,D.H. was manifest early in the course of the disease, at about the time virus shedding ceased.

Inasmuch as the peritoneal exudate cells were pooled for assay, it cannot be stated how each animal responded in terms of D.H. However, while two animals of 13 had significant serum neutralizing antibody levels at four days and two more also had neutralizing antibody at eight days after infection, the remaining animals still had low antibody levels by plaque reduction assay at 12 days. Nonetheless all animals stopped shedding virus within nine of inoculation; and the two animals with apparently high serum antibody levels at four days were still shedding virus at that time.

This evidence is not conclusive, but strongly suggests that D.H. is elicited early in the course of the illness (within six days) and may be sufficient itself or a desirable adjunct to neutralizing humoral antibody in the eradication of virus.

3. Feasibility of a Cell Mediated Cytotoxicity Assay Using Guinea Pig Tracheal Organ Culture - A pilot study was undertaken to evaluate the direct effects of sensitized lymphocytes upon a tissue actively shedding virus to which the lymphocytes had been sensitized.

Lengths of cervical tracheas were excised from ether anesthetized inbred guinea pigs, stripped of connective tissue, quartered longitudinally and each strip divided into fragments two to three mm long. Such fragments remain viable for approximately one week when incubated in plastic petri dishes with 5 to 8

pieces per plate. They actively support the growth of unadapted influenza A2 and parainfluenza 3 viruses. Virus growth reaches a peak in 3 to 4 days at 34°C and then began a slow decline. Maximal virus titers were $10^{6.0}$ to $10^{7.0}$ $TCID_{50}$/ml for influenza and $10^{5.0}$ to $10^{6.0}$ $TCID_{50}$/ml for parainfluenza 3. Electron microscopic examinations of osmium tetroxide stained sections of these tracheal fragments showed virus budding from the limiting membrane of respiratory epithelial cells.

Influenza A2 sensitized spleen cells or peritoneal exudate cells were taken from inbred guinea pigs which had received influenza virus (allantoic fluid harvest) emulsified with H37Ra complete adjuvant subcutaneously two weeks before. At the time of use, a direct M.I.F. assay was performed using presumptively sensitized peritoneal exudate cell pools or sensitized spleen cells combined with normal inbred guinea pigs peritoneal exudate cells. Inhibition of migration in the presence of antigen was interpreted as a manifestation of D.H. in the cell pool used.

Tracheal fragments from guinea pigs of the same strain as those providing the sensitized cells were infected with influenza virus one or 3 days before the sensitized and control unsensitized cells were added to the culture fluids. Culture fluids were harvested daily for quantitation of virus growth. Individual tracheal fragments were also removed from culture, mixed and later stained for light and electron microscopy.

Minor variations in viral growth in cultures containing sensitized lymphocytes versus those containing unsensitized lymphocytes were noted, but the differences were not striking and were of uncertain significance particularly because of the limited resolution of the virus titration technique.

Neither light microscopy nor electron microscopy of tissue specimens taken at various times after addition of cells revealed significant morphologic changes attributable to the added lymphocytes. The direct effects of sensitized lymphocytes upon tissues actively shedding virus remain unresolved.

Significance to Biomedical Research and the Program of the Institute:

It now seems likely that respiratory viral infections do elicit a D.H. response in humans and animals. However, the contribution of this phenomenon to the pathophysiology of such illnesses remains poorly understood. The effective treatment of respiratory viral infections and especially the production of efficacious vaccines require that this understanding be improved.

Proposed Course of Project:

A short survey of children undergoing tonsillectomy is to be completed using the better, indirect M.I.F. assay of D.H. Serum specimens will also be acquired from these patients to provide correlates with the levels of serum antibodies.

More refined data concerning the development of D.H. in the parainfluenza 3 inbred guinea pig system in the first 6 days of illness will be collected, and an attempt to extend these findings to influenza A2 infected guinea pigs will be made.

Honors and Awards: None

Publications: None

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LABORATORY OF VIRAL DISEASES

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PHS-NIH
SUMMARY REPORT

ANNUAL REPORT OF THE LABORATORY OF VIRAL DISEASES, NIAID
July 1, 1971 - June 30, 1972

Dr. Wallace P. Rowe
Chief, Laboratory of Viral Diseases

The program of LVD, while comprised of many independent projects, has a high degree of coherence in being directed toward two major areas of interest: understanding the biology of tumor viruses, and evaluating the host defense mechanisms which are protective and curative for viral infections. In both of these important fields, LVD is in a strong position of scientific leadership.

The tumor virus work has made major strides in the past year; this is particularly gratifying in that our many years' investment of effort in developing techniques and basic knowledge of the laboratory behavior of various oncogenic viruses is now bearing fruit. This is true of both of our tumor virus programs--C-type RNA viruses, and adenovirus-SV40 hybrid viruses.

In the C-type RNA virus studies, we have established several crucial points required for proving that the genomes of these viruses are carried as chromosomal elements of normal cells. Our discovery of the remarkable ability of 5-iododeoxyuridine (IUDR) and 5-bromodeoxyuridine (BUDR) to activate synthesis of murine leukemia virus (MLV) by normal cells of the AKR mouse enabled us to prove that the MLV genome is carried, in unexpressed form, by all AKR cells. Also, by genetic studies of transmission of virus from AKR mice to offspring of crosses with low virus mice, we have demonstrated the presence of two chromosomal loci in AKR, either of which results in the appearance of infectious virus early in life. The location of one of these loci on the genetic map of the mouse has been identified. If we can establish that viral genetic markers segregate with these chromosomal loci, we will have conclusively shown that the virus is a normal cellular genetic element, and preliminary studies indicate that this is the case. Proving this phenomenon is of great potential importance with implications for many biological phenomena and disease processes. With the demonstration by an LVD scientist (Dr. Takemoto) that Visna virus is a close relative of the C-type RNA virus, the finding of C-type viral lysogenicity in normal mammalian cells is already directly applicable to one class of slow virus infections.

The non-defective adeno-SV40 hybrid viruses are rapidly developing into a major tool for the genetic and biochemical analysis of SV40. Since SV40 has become the most intensively studied oncogenic DNA virus, whose analysis is expected to give profound insight into the genetic and molecular basis of viral transformation and the malignant state, the hybrids have assumed great importance. The studies by Drs. Lewis and Kelly have now shown that the five non-defective hybrids all represent successive deletions of the SV40 genome having one end in common and inserted in the same place in the adenovirus genome. Collaborative studies show that each hybrid makes a set of SV40

mRNA molecules corresponding to the SV40 gene content. Since three SV40 gene products or functions produced by the various hybrids have not been identified, the classes of mRNA can be identified with the genes they correspond to. One of the collaborating laboratories is well into the sequencing of two of these SV40 mRNA's made by the hybrids. These deletion mutants have also supplied the information for mapping three SV40 genes and a point on the SV40 DNA molecule where transcription is initiated by E. coli polymerase. These findings represent the great bulk of the present knowledge of SV40 genetics.

Research on interferon and other host defenses against viral infections has continued on a broad front ranging from study of the molecular events to trials in man. LVD staff provides leadership by conducting its own research; by cooperating with the Antiviral Substances Program, NIAID, on contract studies; and by collaborating in studies with other institutions. While animal experiments continue to demonstrate prophylactic and therapeutic control of various viral infections by interferon, successful application of interferon to man is presently impeded by the technical difficulty of safely inducing large enough quantities of interferon in man. To overcome the limitation of safe induction of interferon in man, certain molecular and cellular mechanisms controlling interferon induction and antiviral action were studied. Experimental application of this knowledge to experimental infections of animals indicated efficacy and certain limitations. Early trials in man are being conducted in volunteers and patients with herpes simplex eye infections, respiratory infections, subacute sclerosing panencephalitis, Reye's syndrome, congenital rubella, respiratory diseases and tumors. The early results in humans suggest that (1) present methodology permits safe induction of only low levels of interferon in man, (2) large amounts of interferon can be produced by man but so far only under limited conditions, (3) the low levels of interferon achieved in man provide minimal protection against viral infections. Continuing studies are aimed at (1) better understanding of the factors regulating interferon production and action so as to achieve high levels in man, (2) producing large amounts of interferon, and (3) continuing trials in human disease. A major development has been the commitment by Dr. C. Anfinson (NIAMD) to personally undertake studies of purification of human interferon with the help of the Intramural and Collaborative branches of NIAID. Successful purification could lead to understanding the structure-function relationships of the interferon molecule, to rapid assays and to synthesis.

During the past year, two LVD scientists, Dr. M.D. Hoggan and Dr. K. Takemoto, were honored with the USPHS Commendation Medal.

The summaries of the projects follow.

Activation of murine leukemia virus in cell cultures. All clonal cell lines from virus-negative AKR embryo cell cultures were found to be capable of producing infectious murine leukemia virus either spontaneously or after various experimental manipulations such as X-irradiation or exposure to certain chemicals. The most efficient inducers were the halogenated pyrimidines BuDR and IuDR which gave activation rates of at least one cell in 1000. These findings indicate that all AKR embryo cells growing in cell culture contain the full

viral genome in unexpressed form and provide techniques for studying the mechanism of virus activation and for comparing mouse strains, such as AKR, which produce large amounts of virus and those which produce little or no virus. (Lowy, Rowe, Teich, Hartley).

Genetics of MLV infection. Genetic studies of the transmission of MLV by the high virus producing AKR mouse were based on tests for infectious virus in various F_1, F_2, and backcross generation hybrids of AKR and selected low virus mouse strains of differing Fv-1 and H2 genetic backgrounds. The segregation data support the concept of germ cell transmission of MLV infection by means of a genetic element coding for infectious virus and indicate that the AKR mouse carries two unlinked chromosomal loci, both of which have the capacity to induce virus synthesis early in life. One of the loci is located on linkage group I, 25 to 30 map units from the albino locus; linkage of the other is not known. Single gene families are being established to facilitate linkage testing and a virus host range marker is being utilized to study whether the genes detected represent the viral genome itself or expression genes influencing the activation of viral determinants elsewhere in the cell. (Rowe, Hartley).

Delineation of the SV40 DNA segments carried by nondefective adeno-SV40 hybrid viruses. Studies on the DNA from the 4 new nondefective hybrids show that each contains a small segment of SV40 DNA covalently linked to what appears to be the entire adeno 2 genome, the ND_2 hybrid DNA contains 2.6% SV40 sequences, ND_{3A} DNA 0.2%, ND_{3B} DNA 2.2%, and ND_4 DNA 3.5%. By molecular weight then the ND_2 hybrid carries 570-650,000 daltons of SV40 DNA; ND_{3A} has only 40-50,000, ND_{3B} has 480-550,000 and ND_4 contains 770-880,000 daltons of SV40 DNA. As these hybrids induce only early SV40 antigens and early SV40 RNA, they contain small sequences of the early SV40 genome. (Lewis; Levine, NCI; Henry, U. of Missouri; Oxman, Levin, Harvard).

Location of the SV40 DNA segments within the genome of the nondefective adeno-SV40 hybrid viruses. Examination, by electron microscopy, of heteroduplex DNA molecules formed by annealing nonhybrid adeno 2 DNA with hybrid DNA shows that the SV40 DNA in each hybrid genome is a single linear piece inserted at a point which is 14% of the length of the entire adeno 2 genome from the short end of the heteroduplex molecule. Preliminary measurements indicate that the amounts of SV40 DNA incorporated within the hybrid genome are somewhat larger but in general agreement with the figures obtained by reconstruction. (Kelly and Lewis).

Association of SV40 transplantation antigen and enhancing functions with SV40 DNA segments carried by one or more nondefective adeno 2-SV40 hybrids. Of the 5 nondefective adeno 2-SV40 hybrids under study only the 2 (ND_2 and ND_4) carrying the largest segments of SV40 DNA induce SV40 specific transplantation immunity in hamsters challenged with tumor producing inocula of SV40 transformed hamster cells. Both the ND_2 and ND_4 hybrid induce SV40 resistance as effectively as does wild type SV40 itself. Three (ND_1, ND_2, and ND_4) of the 5 nondefective hybrids induce SV40 U antigen and grow in monkey cells which fail to support the growth of nonhybrid human adenoviruses. Each of these hybrids will enhance the growth of adeno 7 in monkey cells as efficiently as will co-infection with SV40. These studies associate SV40 transplantation immunity with a specific sequence of early SV40 DNA which differs from those sequences

of SV40 DNA which induce SV40 T and U antigens. In addition it appears that the SV40 U antigen (or possibly biochemical events leading to its synthesis) is responsible for the enhancement of human adenovirus growth in monkey cells (Lewis, Crumpacker and Rowe).

Relationship between certain "slow" viruses and oncogenic viruses. Evidence relating visna and progressive pneumonia viruses to the RNA tumor viruses has been accumulated. It includes: (1) ultrastructural studies of isolated virions and their morphogenesis in infected cells revealed close similarities; (2) like the RNA tumor viruses, visna virions contain reverse transcriptase and the 70S RNA molecule is comprised of long tracts of polyadenylic acid; (3) the host range for transformation by these "slow" viruses has been extended to rat and possibly sheep cells. An unexpected finding was that rifamycin and rifampicin, which have been reported to inhibit reverse transcriptase of and focus formation by RNA tumor viruses, enhanced visna virus growth. Studies of the similarities and differences between these and other "slow" viruses to oncogenic viruses will continue with the expectation that the findings may provide insight into the mechanisms of chronic virus diseases (Takemoto).

Induction of Avian Leukosis Virus by IUDR: In view of the ability of IUDR to activate leukemia virus in mouse cells, similar experiments were done in chick embryo cells. IUDR treatment induced avian-leukosis virus (ALV) antigen in a significant proportion of both group-specific antigen (gs)-positive and gs-negative, virus-free, chick embryo cells. In addition, low frequency induction of infectious ALV was observed. These findings confirm recent studies from other laboratories which show that the ALV genome is present in both gs-positive and gs-negative cells, and show that IUDR is as efficient an inducer as any other known inducer for chick cells (Cook).

Host defenses during viral and other infections. Several different approaches were used. Enhancement of polynucleotide induction of interferon by DEAE-dextran was shown to be largely due to the action of DEAE-dextran on the cell plus its ability to increase ribonuclease resistance of polynucleotides. Another basic substance, neomycin, was shown to enhance the interferon inducing effect of small doses of poly I.poly C in rabbit ocular tissue cultures. Other experiments demonstrated that animals with antibody to poly I.poly C produced much less interferon when stimulated with that polynucleotide and that this antibody inhibiting effect of induction also occurred in vitro but to a lesser extent. Since man can be a good producer of interferon, during certain viral infections, and since polynucleotides are relatively poor inducers in man, these studies will be continued with the expectation of increasing the induction of interferon by these inducers in man (Baron, Dianzani, Buckler).

Immunity and immunosuppression during infection. Three clinically important immunosuppressive chemicals were found not to suppress the interferon mechanism in mice. Other experiments employing immunosuppression indicated that the immune response seems to play little or no role in the pathogenesis of scrapie infection. In comparison, the immune response during lactic dehydrogenase virus infection plays a partial role in controlling viremia after the second week of infection. This action was probably due to the antibody response rather than to the cellular immunity response. Studies to dissect the relative

roles of antibody, cellular immunity, and interferon during these infections will continue (Worthington, duBuy, Baron).

The effectiveness of host defenses during a chronic virus infection. Factors controlling the chronic viremia in mice, caused by lactic dehydrogenase virus (LDV), were studied both in vivo, and in vitro in the macrophage-LDV model. The role of antibody was studied in vivo by prolonged treatment of mice with the immunosuppressive drug cyclophosphamide. The drug not only led to the absence of neutralizing antibody against LDV, but also to the absence of sensitized (antibody-bound but infectious) LDV. The absence of antibody apparently resulted in an increased viremic titer of LDV. The relative sensitivity of LDV to interferon was studied in vitro in the LDV-macrophage model, as compared to the sensitivity of two other viruses. This sensitivity to interferon, in decreasing order, was: vesicular stomatitis virus, Sendai virus and LDV. These results indicate that the eventual control of the LDV viremia by interferon treatment will require a sustained high concentration of interferon (duBuy, Worthington, Baron).

Induction of interferon. The induction of interferon by poly I.poly C appears to take place through an interaction of the drug with the cell membrane, where nuclease action causes breakdown of the poly I.poly C. Little or no biologically active poly I.poly C can be found in the cells. Other findings demonstrated that suitable priming of mice with oxidized amylose can lead to a 6 to 100-fold enhancement of interferon production when the mice are stimulated with poly I.poly C. Enhancement also occurs in the cat, whose response to poly I. poly C resembles somewhat more closely that of man (Levy).

Mechanism of antiviral action of interferon. The first interaction with cells of the RNA genome of an infecting virion may be with cytoplasmic proteins, yielding a particle resembling an "informosome" or m-RNP particle. These proteins may be modified in interferon treated cells in such a way that they do not bind effectively to the viral RNA (Levy, Markoff).

Treatment of infections with poly I.poly C. Studies on the effect of poly I. poly C during infections of mice with C. albicans and C. immitis demonstrated increased severity of infection while C. neoformans infection was not altered by treatment. This finding indicates possible hazard in the use of polynucleotide inducers in the treatment of viral infection. Experiments with T. congolense indicated that certain strains of this protozoan were inhibited due to immunological enhancement by the polynucleotide rather than to induction of interferon (Baron, Worthington, Hasenclever, LM, Herman, LPD).

"Persistent AAV infection" of Detroit-6 cells. Mass cultures of the Detroit-6 cell line were infected with AAV-1H and AAV-3H and carried for as many as 70 passages in the absence of adenovirus helper. These cultures will produce AAV when challenged with helper adenovirus and have been shown to carry AAV-DNA in the absence of detectable AAV-antigen. A model system has thus been found for studying the occult AAV-2 infection found in approximately 1% of human embryos and the AAV-4 infection found in 20-40% of African green monkeys (Hoggan, Johnson, LVD;Berns, Johns Hopkins University).

Probable "precursor" proteins of AAV visualized. Antisera made against the three polypeptide constituents of AAV-3H virions stain unique antigens in AAV-infected cells by immunofluorescence. These antigens appear earlier than those stained with antisera prepared against the whole virion, and one first appears in the cytoplasm of infected cells. This technique may well be of great value in studying the development of other viral proteins (Hoggan, Johnson, LVD; Blacklow, University Hospital, Boston, Mass.).

Polypeptide subunits of Haden virus identified. Three distinct polypeptide subunits of the Haden virus have been identified by polyacrylamide gel separation. Thus a sixth member of the parvovirus group has been shown to contain 3 polypeptides which may be an invariable characteristic of this virus group (Hoggan, Johnson, LVD).

Study of biological and chemical properties of amebal viruses. An amebal virus assay system has been developed and a number of properties of one of the viruses have been investigated: growth curve, antibody neutralization, and stability as a function of pH, temperature, freezing, and treatment with a variety of chemicals. We have been unable, despite an intensive effort, to purify the virus due to its tenacious adherence to membranes of lysed amebae. Likewise we have been unable to permanently free HB-301 amebae of its virus despite 35 consecutive passages in the presence of antiserum (Mattern, Hruska, Daniel, Diamond, LPD).

Study of ultrastructural relationships of protozoa and metazoa particularly with respect to locomotor and sensory organelles. A detailed ultrastructural study of Trichomitus batrachorum has led to further clarification of the evolutionary kinships among the trichomonads. Further, pseudocysts of T. batrachorum have revealed an unusual internalization of the recurrent flagellum with dissociation of flagellar microtubules into microfilaments. A comparative study of the human taste buds and insect and nematode contact chemoreceptors have revealed interesting ultrastructural similarities which ultimately may be correlated with physiological mechanisms involved in chemoreception. Ultrastructural changes in the human taste bud have been observed associated with taste abnormalities in Sjogrens syndrome (Mattern, Daniel).

Serial No. NIAID 68H
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic and Other Studies of Animal Leukemia Viruses.

Previous Serial Number: SAME.

Principal Investigator: Dr. M.K. Cook.

Other Investigators: Dr. W.P. Rowe, Miss Johnna Sears.

Cooperating Units: Dr. Calvin G. Aulisio and Mr. William Jahnes (DBS); Division of Research Services, L.A.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	27/12	3/12	0	30/12
Professional:	24/12	0	0	24/12
Other:	3/12	3/12	0	6/12

Project Description:

Objectives: Study of the biology, immunology, and natural history of agents associated with Marek's disease and of Type I (RIF-type) and Type III (RE-type) avian leukemia viruses.

Methods Employed: Tissue culture systems and standard animal inoculation procedures were employed. Current methods were used to define and characterize viruses isolated. Immunofluorescence, complement-fixation, agar-gel diffusion, immuno-electrophoresis techniques, and neutralization tests are employed to define similarities and differences in the antigenic components of avian leukosis viruses. Gradient centrifugation and column chromatography are used to separate antigenic components of strains of Marek's disease virus.

Major Findings: Treatment of group-specific (gs)-positive and gs-negative virus-free chick embryo cells with 5-iododeoxyuridine (IuDR) resulted in induction of avian-leukosis virus antigen in vitro. In addition, infectious avian leukosis virus was recovered from 16.7% of the IuDR-treated gs-negative chick cells and 6.4% of the IuDR-treated gs-positive cells. Fluorescent antibody studies of IuDR-treated cells showed that when infectious virus was not recovered, the presence of fluorescent antigen was sporadic and transient.

These studies confirm previous observations that the viral genome of avian leukosis virus may be present in both gs-positive and gs-negative cells. The findings are similar to those in the murine leukemia system where sporadic and transient induction of fluorescent antigen for murine leukemia virus in IuDR-treated murine cells has been noted and low frequency induction of infectious virus from antigen positive cells has been reported.

Repeated failures in chickens to produce antibodies to the "soluble antigens" detectable in gel diffusion tests of low tissue culture passages of field isolates of Marek's disease herpes virus indicated that chickens could not be used for antibody production; heterologous hosts were required. These findings are reminiscent of the immunologic tolerance for gs antigens of homotypic C-type RNA viruses of mice, chickens, and cats, as well as for LCM virus. After repeated tissue culture passages, however, field isolates which showed conversion to type II plaque forms, as well as JM strains of herpes virus after many tissue culture passages, did produce "soluble antigens" in tissue culture which were capable of eliciting antibodies in homologous hosts. This is the first finding of conversion of the immunologic capacity of a strain of virus associated with tumor production being manipulated in tissue culture and probably acquiring a new antigen or undergoing major antigenic change and at the same time undergoing a change in size of plaques produced in tissue culture, a change from close cell-association to releasing virus into supernatant media, and undergoing a loss in pathogenicity.

Limited studies have shown that lymphocytes cultured from the spleens of infected or hyperimmune chickens and normal chickens react differently in migration-inhibition tests.

Proposed Course of the Project: Efforts will be made to study the immune phenomenon playing a role in Marek's disease. The levels and types of globulin in infected birds will be studied. The active antigenic components of field strains of Marek's disease and laboratory manipulated strains will be separated and compared and correlated with immunologic tolerance and failure of tolerance. Further studies of lymphocytes from spleens of infected or hyperimmune chickens and normal birds will be carried out.

Significance to Biomedical Research and the Program of the Institute: Mechanisms of cellular immunity studied by using the Herpes-type virus of Marek's disease may help to resolve some of the problems in tumor regression and hypersensitivity reactions. Candidate viruses for the etiologic agent(s) of Marek's disease offer models for the study of lympho-proliferative and tumor viruses in man and animals. The effect of contamination by such agents in viral vaccines prepared in tissue cultures of avian tissue must also be assayed.

Serial No. NIAID 71A
1. Viral Oncology
2. Viral Diseases
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Parvoviruses with Emphasis on the Adenovirus-Associated Viruses (AAV).

Previous Serial Number: SAME.

Principal Investigators: Dr. M. David Hoggan,
Dr. F. Brent Johnson.

Other Investigators: Dr. Wallace P. Rowe and Gunter F. Thomas.

Cooperating Units: (1) Dr. William T. London, PR/NINDS.

(2) Dr. Kenneth I Berns, Johns Hopkins School of Medicine, Baltimore, Maryland; Dr. Neil R. Blacklow, University Hospital, Boston, Massachusetts.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	20/12	0	12/12	32/12
Professional:	20/12	0	12/12	32/12
Others:	0	0	0	0

Project Description:

Objectives: To study the many similar biological, immunological, biophysical, and biochemical properties of the parvoviruses in order to better understand their host parasite relationships in various in vitro cell systems, thus gaining insight to the diseases they cause in their natural hosts.

Methods Employed: Various parvoviruses are grown in appropriate in vivo hosts or in vitro cell culture systems, purified by conventional means such as isopycnic gradient centrifugation, and their properties determined.

Purified virus preparations are disrupted by sodium dodecyl sulfate (SDS) and their constituent polypeptides separated by polyacrylamide gel electrophoresis. Antisera are then prepared against the individual polypeptides by footpad inoculation of guinea pigs with complete Fruend adjuvant.

Detroit-6 cells are inoculated with purified AAV at high multiplicity (100 to 10,000 $TCID_{50s}$ per cell) in the absence of adenovirus helper. Such "infected" cell populations are then split 1:400 once each week and tested for the presence of AAV with and without added helper virus.

Virus is measured by conventional methods including complement fixing antigen induction, immunodiffusion, electronmicroscopy, immunofluorescence, hemagglutination, and DNA-DNA in vitro hybridization.

Major Findings: Previous studies had indicated that AAV-replication occurred in Rhesus monkeys inoculated in utero only when simultaneously inoculated with adenovirus (SV-15) as helper. Fetuses inoculated with AAV without helper developed normally; AAV virus could not be recovered from them at the time of birth and they showed no serological evidence of AAV infection. AAV could not be induced by challenge with helper virus; however, such monkeys were susceptible to co-infection with AAV and helper with the development of antibody to both. In all previous studies all fetuses receiving helper adenovirus died within a few days, although many were retained by the mother until term. Recent attempts to reduce the adenovirus inoculation low enough to produce infection with AAV without fetal death were not successful. AAV replicated (as measured by antibody development in the mother) only when adenovirus replicated. When adenovirus replicated, the infection proved fatal to the fetus whether AAV was present or not. These data were not too surprising in light of our epidemiological data in humans and primates indicating little or no AAV replication in vivo without adenovirus replication.

On the other hand, since approximately 1% of all human embryonic kidney cell cultures we receive in our laboratory are occultly infected with AAV-2 and 20-40% of African green monkey kidney cultures are infected with AAV-4, it seems apparent that AAV must be able to persist in some state in the absence of helper virus, and is probably transmitted vertically.

The development of the AAV-Detroit-6 cell system in which it has been possible to detect AAV in cells after as many as 70 passages in the absence of helper has provided an excellent model for studying this problem.

It has now been possible to establish both AAV-1 and AAV-3 "persistently infected" carrier lines of Detroit-6 cells, and some of the parameters necessary for their establishment have been determined. Thirty-six cell clones from the AAV-1 "infected" Detroit-6 mass carrier culture have been established and one of these clones produces large amounts of CF antigen and infectious AAV when challenged with adenovirus helper. When coverslip preparations of this clone are stained with AAV-1 antiserum in the immunofluorescence test, antigen is found in 90-100% of cells infected with adenovirus or herpes simplex virus helper, while unhelped preparations are negative for AAV-1 antigen. On the other hand, studies carried out in collaboration with Dr. K. Berns using in vitro hybridization techniques indicate that AAV DNA can be detected in the positive clone in the absence of helper virus while it is missing in control passage Detroit-6 cells which have not been exposed to AAV.

In addition to the single clone which produces infectious AAV and CF antigen when challenged with adenovirus helper, a second clone has been shown to produce FA stainable antigen when challenged with helper virus, but no infectious virus or CF antigen can be demonstrated. It, therefore, appears that only part of the AAV-1 genome is "integrated" in this clone.

Our attempts to isolate clones of AAV-3 "infected" cells from the AAV-3 infected mass culture of Detroit-6 cells have produced 28 negative cultures. It thus appears that few cells in a mass culture of Detroit-6 cells become infected but these few cells can produce enough progeny to be carried for greater than 70 passages.

An AAV strain has been reported by Domoto and Yanagawa from the canine adenovirus (infectious canine hepatitis). We have grown this virus in primary canine kidney cells co-infected with ICH virus, purified it by banding CsCl isopycnic gradients and have found it does not react with antiserum of any of the known AAV types. Antiserum has been prepared and reciprocal testing indicates it is unrelated to the four previously described types of AAV.

The polypeptides of Haden virus have been studied in some detail. It has been found that Haden virus contains one major and two minor polypeptides as has been similarly reported for AAV, RV, and H-1, the major polypeptide having a MW of approximately 65-70,000, and comprising about 80% of the total virion protein. Preliminary studies of empty virions (lacking DNA) demonstrate the same three polypeptides in about the same concentration as are found in the full band.

Antisera have been prepared against the three constituent polypeptides (VP-1, VP-2, and VP-3) of AAV-3H. These antisera have been characterized by CF, immunodiffusion and immunofluorescence. In immunofluorescence tests (FA) these antisera stained heat stable antigens with distinct morphologies in cells co-infected with either adenovirus or herpes simplex virus as helper. VP-1 antisera stained cytoplasmic antigens at 14 hours after infection and both cytoplasmic and nuclear antigen by 18 hours. VP-2 antisera stained discreet globular areas in the nucleus beginning at about 14 hours and VP-3 antisera stained nearly the entire nucleus. The VP antisera all stained antigens which appeared about 2 hours before antigens that were stained with antisera prepared against the whole virion. In CF and immunodiffusion tests the polypeptide antisera and whole virion antisera did not crossreact with each other, which strongly suggests that unique antigens are revealed by SDS treatment which can be detected in untreated cells prior to their polypeptide incorporation into the whole virion.

Significance to Biomedical Research and the Program of the Institute: The number of parvoviruses is rapidly increasing as new techniques and better understanding of their biology is developed. These small 20-30 nm viruses which contain single-stranded DNA remain an enigma because of their extreme stability (heat 60° or 70°) and limited content of genetic information (less than 2×10^6 MW DNA). Their propensity to become "integrated" in their hosts

in some cases throughout life (Rat Virus) and apparent vertical passage in nature in an occult state makes them ideal candidates for models in the modern era of "genetic engineering" and they remain prime candidates for such diseases of unknown etiology as infectious hepatitis, acute gastroenteritis, certain types of arthritic conditions and various neurological syndromes.

Proposed Course of the Project: Comparative studies of various parvoviruses will continue both at the biological and molecular level.

Specific short term goals for the coming year include: (1) To complete analyses and write up the studies conducted during the past two years on AAV *in utero* infection of the Rhesus monkey. (2) To investigate the effect of concomitant parvovirus infection on hepatitis produced in hamsters by equine herpes virus infection. (3) To further exploit the finding that parvovirus polypeptides prepared by sodium dodecyl sulfate treatment of the virion with subsequent preparation by polyacrylamide gel electrophoresis can be used to define various precursor states of antigen development. (4) To investigate in detail the already developed *in vitro* model system of persistence of the AAV genome in Detroit-6 cells, and study possible ways of inducing viral replication such as by IUDR treatment, UV irradiation, etc., in the absence of helper virus.

Honors and Awards:

Dr. M. David Hoggan: Recipient of PHS Commendation Medal in 1971 for systematic research investigation to identify and characterize the parvoviruses and their possible relation to disease.

Invited Lectures and Seminars:

Dr. M. David Hoggan:

1. Institut fur Microbiologie und Infektionskrank Ludvig-Maximillians, Universitat Munchen, Germany.

2. Laboratory de Microbiologia Institute Superiore de Sanita, Rome, Italy.

3. Institute fur Hygiene und Mediziniske Microbiologie Universitat, Bern, Switzerland.

4. 1st Conference of Parvoviruses, Oct. 21, 22, 1971, Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont.

5. Department of Microbiology, Cornell School of Veterinary Medicine, Ithaca, New York.

Dr. F. Brent Johnson:

1. Department of Microbiology, Brigham Young University, Provo, Utah.

2. First Conference on Parvoviruses, Oct. 21, 22, 1971, Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont.

Publications:

Blacklow, N.R., Hoggan, M.D., Sereno, M.S., Brandt, C.P., Kim, H.W., Parrot, R.H., and Chanock, R.M.: A seroepidemiologic study of adenovirus-associated virus infection in infants and children. Amer. J. Epidem., 94: 359-366, 1971.

Johnson, F.B., Ozer, H.L., and Hoggan, M.D.: Structural proteins of adenovirus-associated virus type 3. J. Virol., 8: 860-863, 1971.

Johnson, F.B., Blacklow, N.R., and Hoggan, M.D.: Immunologic reactivity of antisera prepared against the sodium dodecyl sulfate treated structural polypeptides of adenovirus-associated virus. J. Virol., in press.

Hoggan, M.D. and Thomas, G.F.: Australia antigen. In Dalton and Haguenau (Eds.): Virus Atlas, New York, New York, Academic Press, in press.

Serial Number NIAID/NCI 71 D
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Mouse Leukemia Viruses

Previous Serial Number: Same

Principal Investigators: Dr. Janet W. Hartley, LVD, NIAID
Dr. Wallace P. Rowe, LVD, NIAID

Other Investigators: Dr. Natalie M. Teich, LVD, NIAID
Dr. Douglas R. Lowy, LVD, NIAID

Cooperating Units: Inside NIH:
Dr. Arthur S. Levine, MB, NCI

Outside NIH:
Dr. John C. Parker, Microbiological Associates, Inc., Bethesda, Md.
Dr. Edward A. Boyse, Sloan-Kettering Institute for Cancer Research, New York, N. Y.

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	133/12	0	0	133/12
Professional:	47/12	0	0	47/12
Others:	86/12	0	0	86/12

Project Description:

Objectives:

To utilize sensitive virus isolation and quantitation procedures for murine leukemia and sarcoma viruses in studying the natural history of infection with these agents and the biology of their growth in tissue culture.

Methods Employed:

Tissue culture procedures, using plaque and focus assays and complement fixing antigen induction; complement fixation; fluorescent antibody techniques; tumor induction in mice, hamsters and rats.

Major Findings:

Activation of murine leukemia virus (MLV) in cell cultures: Further studies with MLV-negative cell lines (derived from small numbers of embryo cells from the high virus AKR mouse strain) indicated that all AKR embryo cells growing in cell culture contain the full viral genome in an unexpressed form. This may be concluded from the demonstration that three such cell lines and all of 18 clones or subclones obtained from two of the lines have the capacity to produce infectious virus either spontaneously or after various types of manipulation such as X-irradiation, SV40 transformation, or treatment with certain chemicals. Spontaneous activation rates were of the order of 1 in 10^8 or 10^9 cells, while X-irradiation increased the rate to 1 in 10^5 cells. In contrast, exposure of the cells to the thymidine analogs 5-bromodeoxyuridine (BUDR) and 5-iododeoxyuridine (IUDR) increased activation rates dramatically with at least 1 cell in 1000 in some lines producing virus six days after treatment with IUDR. Induction of MLV by BUDR and IUDR appears to require incorporation of the analogs into DNA, since addition of 5-fluorodeoxyuridine at the time of exposure to BUDR or IUDR as well as exposure of BUDR treated cells to visible light increased the proportion of activated cells. Thymidine treatment prevented induction. By use of quantitation procedures involving appropriate cell and IUDR titrations it has been possible to detect induction rates as high as one virus-producing cell in 200. Kinetic studies of IUDR activation indicate that some cells are producing virus within 48 hours after initial exposure to the drug, with the number increasing markedly by 72 to 96 hours. The extent of activation of viral gene expression is greater than is indicated by the number of virus producing cells, since 10 to 30 times as many cells are positive for immunofluorescent stainable antigen at three days after exposure to IUDR.

Primary AKR embryo cells are activated by IUDR with an efficiency approximating that of AKR cell lines, and embryo cells derived from crosses of AKR with BALB/c mice (a B-type mouse, whose cells are resistant to the N-tropic AKR virus) are activated almost as efficiently.

In tests for IUDR inducible immunofluorescent stainable antigen and infectious virus in low leukemia incidence mice we have found that continuous lines of embryo cells from BALB/c and DBA mice are induced to form antigen in a large number of cells, but with only minimal production of infectious virus. Neither antigen nor virus has been induced in primary or secondary embryo cell cultures from these strains; negative results have also been obtained with secondary and continuous NIH Swiss embryo lines and a continuous wild mouse embryo line.

Quantitation of virus in AKR mice: Using the XC plaque procedure, the titer and organ distribution of MLV was determined in AKR mice of various ages. Infectious virus first appeared shortly before or after birth, and was continuously present thereafter. Highest infectivity titers were found in uterus and bone, with spleen slightly lower; titers in normal thymus were low, increasing significantly with development of thymic lymphoma. The level of viremia decreased after the first month of life but increased sharply in lymphomatous mice.

Genetics of MLV infection: In an attempt to determine the form in which genetic information coding for infectious MLV is transmitted, the appearance

of infectious virus has been traced in the progeny of crosses and backcrosses of AKR mice with various N- and B-type partners, with different H_2 alleles. The results of this and other studies support the concept of germ cell transmission of a genetic element coding for infectious virus. The data thus far indicate that the AKR mouse contributes two unlinked genes, either of which gives a positive phenotype.

MLV in C57 BL mice: From the B-type C57 BL mouse strain, 5 isolates of N-tropic virus have been recovered, one from a 12 mos. old animal and four from mice 27 mos. old. Only B-tropic virus had previously been found in this strain.

Serology of MLV isolates from wild mice: Preliminary results of tests on two MLV isolates obtained from wild mice indicate that these agents are serologically distinct from viruses of the FMR or Gross-AKR subgroups.

Significance to Biomedical Research and the Program of the Institute:

The finding that IUDR and BUDR are highly efficient inducers of MLV in AKR cell lines provides a powerful tool for studies of leukemia virus infections in mice and other species. In addition to testing for activation of complete infectious virus, induction of synthesis of various viral proteins in negative cell lines can be studied, and it may be possible to utilize the technique in tests for oncogene expression.

Genetic studies of MLV infection have provided strong evidence that information for infectious virus is transmitted chromasomally. Establishment of the location of viral genes should make it possible to determine the relation of the virus loci to other loci coding for MLV-related antigens, and to study the role of genetic factors in determining tumor development.

Proposed Course:

The IUDR activation system will be utilized to explore further the qualitative and quantitative differences between high and low leukemic strains of mice. Studies of the mechanism of the activation process will be continued, with particular interest lying in the effect of cell synchronization, dose response curves, and tracing of biochemical events during activation.

Attempts will be continued to enhance activation rates and to apply more efficient procedures to cell cultures of human origin.

Genetic studies will emphasize the establishment and characterization of single gene families for use in linkage studies of the viral genes and other leukemia-associated genes.

Publications:

Pincus, T., Hartley, J.W., and Rowe, W.P.: A major genetic locus affecting resistance to infection with murine leukemia viruses. I. Tissue culture

studies of naturally-occurring viruses. J. Exp. Med. 133: 1219-1233, 1971.

Pincus, T., Rowe, W.P., and Lilly, F.: A major genetic locus affecting resistance to infection with murine leukemia viruses. II. Apparent identity to a major locus described for resistance to Friend murine leukemia virus. J. Exp. Med. 133: 1234-1241, 1971.

Hartley, J.W., Rowe, W.P., and Wilsnack, R.E.: Detection of murine leukemia virus antigens. Proceedings, Tenth International Cancer Congress, May 1970, Houston, Texas, in press.

Levy, J.A. and Rowe, W.P.: Lack of requirement of murine leukemia virus for early steps in infection of mouse embryo cells by murine sarcoma virus. Virology 45: 844-847, 1971.

Rowe, W.P.: The kinetics of rescue of the murine sarcoma virus genome from a non-producer line of transformed mouse cells. Virology 46: 369-374, 1971.

Lowy, D.R., Rowe, W.P., Teich, N., and Hartley, J.W.: Murine leukemia virus: high frequency activation in vitro by 5-iododeoxyuridine and 5-bromodeoxyuridine. Science 174: 155-156, 1971.

Rowe, W.P., Hartley, J.W., Lander, M.R., Pugh, W.E., and Teich, N.: Non-infectious AKR mouse embryo cell lines in which each cell has the capacity to be activated to produce infectious murine leukemia virus. Virology 46: 864-874, 1971.

Rowe, W.P., and Pincus, T.: Quantitative studies of naturally occurring murine leukemia virus infection of AKR mice. J. Exp. Med. 135: 429-436, 1972.

Rowe, W.P., Lowy, D.R., Teich, N., and Hartley, J.W.: Some implications of the activation of murine leukemia virus by halogenated pyrimidines. Proc. Nat. Acad. Sci., in press.

Serial No. NIAID 71E
1. Viral Diseases
2. Viral Oncology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Viral Antigens in Virus-induced Tumors.

Previous Serial Number: SAME

Principal Investigators: Drs. Andrew M. Lewis, Jr. and Wallace P. Rowe.

Other Investigators: Dr. Thomas J. Kelly, Jr.

Cooperating Units: Dr. Norman P. Salzman et al., (LBV/NIAID).
Drs. Arthur Levine, Lowell Schnipper, Cepheus Patch, and Stuart Siegel (Medicine Branch, NCI); Michael Oxman, Myron Levin (Harvard Medical School); Patrick Henry, Richard Samaha (University of Missouri School of Medicine), and Sherman Weissman (Yale University School of Medicine).

Man Years:

	NIAID Lab Staff	Other NIH Personnel	Guest Workers	TOTAL
Total:	49/12	12/12	0	61/12
Professional:	25/12	0	0	25/12
Other:	24/12	12/12	0	36/12

Project Description:

Objectives: To use the adenovirus-SV40 hybrid viruses as tools to study the genetics of DNA tumor viruses; to define the role of viral genes and antigens in the oncogenic process; to study the biology of adenovirus-SV40 hybrid viruses.

Methods Employed: Tissue culture, fluorescent antibody, complement fixation, serologic epidemiology, and nucleic acid homology techniques.

Major Findings: Adenovirus-SV40 hybrid viruses. Several years ago we described the first nondefective adenovirus-SV40 hybrid designated $Ad2^{+}ND_1(ND_1)$; this virus contains about 250,000 daltons of SV40 DNA inserted into what appears to be the entire Ad2 genome. Due to its nondefective nature, it replicates without helper viruses in both human and monkey kidney cells. This hybrid does not induce SV40 T antigen but does induce the early SV40 U antigen which is heat stable and serologically different from the heat labile SV40 T antigen. The SV40 specific RNA produced by the ND_1 hybrid is only a portion of the RNA

species produced by SV40 early in its replicative cycle prior to synthesis of viral DNA. The ND_1 hybrid has been useful in the genetic analysis of SV40 in that it has permitted the identification of SV40 U antigen and has allowed us to associate this SV40 specific antigen with a small segment of SV40 DNA and a particular species of early SV40 RNA.

More recently we have isolated and begun to characterize four additional nondefective adenovirus-SV40 hybrids. These new agents have been designated $Ad2^{+}ND_2$ (ND_2), $Ad2^{+}ND_{3A}$ (ND_{3A}), $Ad2^{+}ND_{3B}$ (ND_{3B}), and $Ad2^{+}ND_4$(ND_4). They differ from each other and from the ND_1 hybrid in their ability to induce SV40 antigens and SV40 RNA and to grow in African green monkey kidney cells (AGMK).

The ND_2 virus grows efficiently in both human embryonic kidney (HEK) and AGMK cells. Like the ND_1 hybrid it does not induce SV40 T or V antigen, but does induce SV40 U antigen which is detectable by both complement fixation and immunofluorescence. In contrast to the perinuclear distribution of SV40 U antigen in cells infected with ND_1, the SV40 antigen in cells infected with ND_2 is present both in the cytoplasm and perinuclear region. By RNA-DNA hybridization-competition, the SV40 RNA induced by ND_1 competes with about 40% of the SV40 RNA induced by ND_2. Thus the ND_2 virus induces several more species of SV40 RNA than the ND_1 hybrid.

The ND_{3A} hybrid grows only in HEK cells and induces no serologically detectable SV40 antigens. It does induce SV40 specific RNA in infected cells and this RNA competes with only 40% of the SV40 RNA sequences present in cells infected with ND_1.

The ND_{3B} hybrid is similar to the ND_{3A} virus in that it grows only in HEK cells and fails to induce SV40 antigens. However, the SV40 RNA species induced by ND_{3B} compete completely with both ND_1 and ND_{3A} RNA and with 80% of the SV40 RNA species induced by the ND_2 hybrid. Thus it induces more species of SV40 RNA than either ND_1 or ND_{3A} even though there are no SV40 specific functions associated with these RNAs.

The ND_4 hybrid grows in both HEK and AGMK cells and induces both SV40 T and U antigen. By immunofluorescence only intranuclear staining is detected in ND_4 infected cells. The ND_4 hybrid then is similar to wild type SV40 and unlike ND_1 and ND_2 in that the SV40 U antigen it induces is detectable in the nucleus of infected cells. The SV40 RNA sequences induced by ND_4 compete completely with all the SV40 RNA sequences induced by the other nondefective hybrids. These RNA sequences induced by ND_4 seem to represent all of the SV40 RNA sequences induced early by SV40 prior to the replication of viral DNA.

Reconstruction experiments using the DNA from the other four nondefective hybrids indicate that ND_2 DNA contains 2.6% SV40 DNA sequences, ND_{3A} DNA 0.2%, ND_{3B} DNA 2.2%, and ND_4 DNA 3.5%. Studies on the DNA of these new hybrids demonstrate that each is a linear duplex containing covalently linked SV40 DNA and that all are similar to nonhybrid adeno 2 DNA in G + C content (54-57%) and molecular weight (approximately 22-25 X 10^6 daltons). From this data then ND_2 contains 570-650,000 daltons of SV40 DNA; ND_{3A} has only 40-50,000,

ND_{3B} has 480-550,000, and ND_4 contains 770-880,000 daltons of SV40 DNA.

Using electron-microscopic mapping techniques preliminary measurements of heteroduplexes between nondefective hybrid and nonhybrid Ad2 DNA support the estimations of the amount of SV40 DNA incorporated within the genomes of the five nondefective hybrids. Additionally, these studies indicate that the SV40 DNA in each hybrid genome is a single linear piece inserted approximately .14 Ad2 map units from one end of the genome (1 map unit equals the length of the Ad2 DNA).

Hybridization competition studies comparing the SV40 specific RNA induced by ND_4 with early and late SV40 RNA indicate that the SV40 DNA sequences incorporated within the ND_4 genome came from that segment of the SV40 genome which is transcribed early prior to viral DNA replication.

Studies have been completed testing the ability of both defective and nondefective Ad2-SV40 hybrids to induce SV40 specific transplantation immunity in hamsters challenged with SV40 transformed hamster kidney cells. These data demonstrate that of the 8 hybrids tested, only 4 ($Ad2^{++}HEY$, B55, ND_2 and ND_4) induce SV40 transplantation resistance in immunized hamsters.

Immunization with each of these viruses produced a 14-40 fold increase in resistance to tumor induction in immunized hamsters compared with a 20-50 fold increase in resistance induced by SV40 itself. Containing the infectious SV40 genome and 250,000, 50,000, and 500,000 daltons of SV40 DNA respectively, the $Ad2^{++}LEY$, ND_1, ND_{3A}, and ND_{3B} failed repeatedly to induce detectable SV40 transplantation resistance in immunized animals.

Studies testing the ability of the nondefective hybrids to enhance the growth of nonhybrid Ad7 in monkey cells demonstrate that only those hybrids which induce SV40 U antigen and plaque and grow efficiently in monkey kidney cells overcome the abortive replication of Ad7 in this host. ND_1, ND_2, and $Ad2^{+}ND_4$ produce a 100 to 1000 fold increase in the number of infectious Ad7 virions in co-infected monkey kidney cultures in 36 hrs. SV40 has a similar enhancing effect, while nonhybrid Ad2, ND_{3A}, and ND_{3B} virions in co-infected cultures had no detectable effects on the yield of Ad7 virions. A monkey adapted Ad2 mutant which grows and plaques efficiently in monkey kidney cells appears to enhance Ad7 growth in AGMK cells as efficiently as does SV40 and ND_1, ND_2, and ND_4. Consequently, the enhancing ability of ND_1, ND_2, and ND_4 could be due to a mutation in the Ad2 genome rather than an acquisition of a unique piece of SV40 DNA. However, the lack of enhancement by the $ND3_A$ and $ND3_B$ hybrids, which are close relatives of ND_1, argue against this possibility.

Thus there appear to be incorporated within the genomes of the 5 nondefective Ad2-SV40 hybrids under study, 5 overlapping segments of that portion of the SV40 genome which is transcribed early during lytic infection prior to viral DNA replication. We have associated the induction of 3 early SV40 antigens (T antigen, U antigen, and transplantation rejection antigen) with specific segments of this SV40 DNA and with the species of SV40 RNA transcribed from

them. The data on the ND_2 hybrids suggest that those sequences of SV40 DNA responsible for U antigen and transplantation antigen induction are adjacent. The SV40 DNA segment responsible for induction of SV40 T antigen could lie either next to the U inducing region or the transplantation rejection inducing region. Examination of heteroduplexes between several of the nondefective hybrids indicate that the T inducing region is adjacent to the transplantation rejection region.

Replication of SV40 DNA: Studies on the replication of the circular SV40 DNA demonstrated unique structure--eighty to ninety percent of the replicating molecules contained two branch points, three branches, no free ends, and a super helix in the unreplicated portion of the molecule. When the replicating DNA molecules were banded by centrifugation in ethidium bromide-cescium chloride gradients they were found distributed at all densities between that of super-coiled double-stranded DNA (component I) and circular SV40 DNA containing single strand nicks (component II). An inverse correlation was found between the extent of replication of the SV40 DNA and the banding density in ethidium bromide-cescium chloride gradients. By banding the replicating DNA in alkaline gradients (which results in very fast sedimentation rates for covalently closed SV40 DNA circles), it was found that the sedimentation rates decreased as replication progressed. These findings suggested several models for the replication of circular viral DNA.

Significance to Biomedical Research and the Program of the Institute: SV40 is becoming recognized as a genetic tool for understanding certain basic cellular functions, especially the regulatory mechanisms involved in cell division. A number of major laboratories are denoting a significant amount of money and personnel to study SV40 and its interaction with mammalian cells. Biologically defined sequences of SV40 DNA and RNA such as we have in these 5 nondefective Ad2-SV40 hybrids will be very useful in these studies.

The isolation of hybrids containing different sequences of SV40 DNA suggests that recombination between the genomes of different animal viruses may be a more frequent event than previously suspected. Such recombinational events must be considered when screening host range mutants for vaccine production and could explain some of the antigenic variations in animal viruses. The presence of such recombinants among other animal viruses would be useful for genetic studies.

Proposed Course of the Project: Studies are under way to locate these sequences of SV40 DNA on the SV40 chromosome, and, if possible, to determine which of these SV40 DNA sequences are responsible for inducing cellular DNA synthesis. We are also studying their ability to produce tumors in hamsters and to transform hamster and human cells *in vitro*.

Collaborative studies are under way to isolate the SV40 DNA segment from the DNA of the $Ad2^+ND_1$ hybrid; to sequence the SV40 RNA transcribed from the $Ad2^+ND_{3A}$, $Ad2^+ND_1$, $Ad2^+ND_{3B}$, and $Ad2^+ND_2$ hybrids, and to characterize the

products of Haemophilus influenzae restriction enzyme cleavage of SV40 DNA by hybridization with nondefective hybrid DNA.

Invited Addresses:

Andrew M. Lewis, Jr. - 1. Stanford University
2. Johns Hopkins University

Thomas J. Kelly, Jr. - 1. Johns Hopkins University
2. Cold Spring Harbor Laboratory
3. Harvard University

Publications:

Kelly, T.J., Jr., and Rose, J.A.: SV40 integration site in an adenovirus 7-SV40 hybrid DNA molecule. Proc. Nat. Acad. Sci., 68: 1037-1041, 1971.

Oxman, Michael N., Levine, Arthur S., Crumpacker, Clyde S., Levin, Myron J., Henry, Patrick H., and Lewis, Andrew M., Jr.: Studies of nondefective adenovirus 2-simian virus 40 hybrid viruses. IV. Characterization of the simian virus 40 ribonucleic acid species induced by wild-type simian virus 40 and by the nondefective hybrid virus, $Ad2^{+}ND_{1}$. J. Virol., 8: 215-224, 1971.

Sebring, E.D., Kelly, T.J., Jr., Thoren, M.M., and Salzman, N.P.: Structure of replicating simian virus 40 deoxyribonucleic acid molecules. J. Virol., 8: 478-490, 1971.

Serial No. NIAID-71-F
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Interferon and Interferon Inducers

Previous Serial Number: SAME

Principal Investigator: Dr. Hilton B. Levy

Other Investigators: Dr. Louis Markoff, LVD, NIAID
Mr. Freddie Riley, LVD, NIAID

Cooperating Units: Dr. Werner Braun, Rutgers University, New Jersey
Dr. Jeffrey Duenwald, Washington State University, Pullman, Washington

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	4			4
Professional	3			3
Other	1			1

Project Description:

Objectives

To study the molecular and physiological mechanism of the antiviral and antitumor action of polyinosinic.polycytidylic acid and interferon. To participate in obtaining best conditions for testing interferon inducers in man. To study the mechanisms of induction of interferon synthesis.

Methods Employed

Many biochemical methods have been employed. Included, but not all inclusive, are spectrophotometry, single and double

isotope tracing, gel electrophoresis, gradient centrifugation, paper and column chromatography, etc. In addition, various biological techniques have been employed, both in tissue culture and in animals, as indicated in the sections on results. Several extensions of the intramural program have been developed in collaborative programs through the contract branch of NIAID.

Major Findings

For convenience of presentation, results obtained during the past year will be divided into several sections, even though many of these are closely interrelated.

Induction of Interferon.--Studies have been initiated to gain information about the factors that induce cells to synthesize interferon; specifically in response to poly I.poly C. We have attempted to determine how much poly I.poly C is taken up by cells. When cells are exposed to poly I.poly C at varying drug concentrations and for varying times, we cannot recover any biologically active poly I.poly C from the cells, using extraction and assay methods that we have shown to be effective and sensitive. If cells are exposed to radioactive poly I.poly C, on the other hand, one can indeed find intracellular radioactivity, but the radioactivity represents broken down poly I.poly C that has been reincorporated into cellular RNA. If one works with isolated L cell membranes, one is indeed able to show some association of the radioactivity of the poly I.poly C with the membranes, but the amount of membranes used represents a very large number of cells. This association is enhanced by the presence of DEAE-dextran, just as is the interferon inducing capacity of poly I.poly C. In addition the membranes have the capacity to hydrolyze poly I.poly C. Taken together, these data suggest that the induction of interferon by poly I.poly C involves an interaction of the agent with the cell membrane, followed by rapid hydrolysis and that it is this interaction that induces the cell to make interferon.

We have found that Amylose into whose structure carboxyl groups have been introduced by oxidation (compound is referred to as COAM) when administered at a proper time prior to poly I.poly C increases the amount of serum interferon induced by the drug by a factor ranging from 6-fold to 100-fold. (The compound COAM was discovered by workers at the University of Leuven). This has been observed in mice and in cats (with Dr. Duenwald) and is currently under test in other species including primates. The intent is to test the combination in man if results so indicate. COAM is a very potent immunoenhancer as measured by the Jerne

plaque assay (with Dr. Werner Braun). COAN causes a number of biochemical effects that are currently under study.

There has long been the question as to exactly which of the events in virus replication triggers the interferon response. Is it the input virion; the progeny single stranded nucleic acid or the double stranded forms found during virus replication. Inconclusive data have been advanced for all three possibilities. We have developed information which suggests that the input virion is effective per se. The reasoning behind these experiments was the following. If cells are pretreated with interferon prior to infection with a suitable virus such cells can produce new interferon earlier and in larger quantities than cells not so pretreated. This phenomenon is known as priming. We have now shown that non-primed cells, when infected with Chikungunya virus, produced the several single-stranded and double-stranded species of viral RNA characteristic of this group of viruses about concurrently with the production of interferon. On the other hand, those cells that have been primed by pretreatment with interferon produce virtually no new viral RNA, either single- or double-stranded, even though they produce more new interferon than the non-primed cells. These data strongly indicate that new viral RNA synthesis is not needed for interferon production and thus the input virion may be the inducing entity.

Induction of the Virus Resistant State--Previous studies have shown that interferon does not exert any discernable effect on rates of total cell RNA synthesis, but alterations in minor RNA species would not have been detected. We have found that interferon induces alteration in the messenger ribonucleoprotein (RNP) particles both those found on polysomes where they are presumably acting as messenger RNAs, and those found in the postribosomal cell fraction--the so-called "informosome" fraction. Some preliminary experiments suggest that a large part of the differences between the RNP particles from control and interferon treated cells resides in the proteins rather than in the differences in RNA. The differences are surprisingly extensive.

Mechanism of Antiviral Effects of Interferon--Work is continuing on the nature of the 50S particle that appears to represent the first interaction between the input Mengo virion and the cell--the particle whose formation is blocked by the action of interferon. We find also that there is a 100S particle containing the input virion RNA. Both of these particles may represent the combination of the parental virion RNA with the type of proteins found in the "informosome" messenger RNP particles described in the previous section.

A number of effects of interferon preparations are being found in addition to its antiviral action. We find in experiments with Dr. Werner Braun that interferon preparations act as immunoenhancers for the formation of humoral antibodies, by a mechanism that may involve the cyclic AMP system. However, there are suggestions that the immune enhancing properties of interferon preparations reside in a 2 component system rather than just in the interferon itself.

COAM which, as just mentioned, is a potent enhancer of interferon production also is an extremely effective immunoenhancer.

Significance to Biomedical Research and the Program of the Institute

The relevance of this project has two aspects. Some of the work such as that with COAM and some other studies dealing with clinical stabilization of poly I.poly C against the hydrolytic action of human serum enzymes could lead to successful therapy of viral disease in man. The other is more theoretical molecular biology and deals with such matters as control of protein synthesis at the translation level; the molecular and membrane events in induction of protein synthesis; and the relationship of the immune system to the interferon system. Both of these aspects are directly in line with the Institute's program to understand viral pathogenesis and to control it in man.

Proposed Course of the Project

The work on enhancing interferon production will be tried in other species and hopefully in man. The role of the cellular proteins that may selectively control messenger RNA translation will be investigated further and the relationship between interferon production and antibody production will be further studied.

Honors and Awards:

The work reported here has been selected for inclusion in the M. D. Anderson Annual Year Book on Cancer.

Member Organizing Committee, International Interferon Workshop, 1972.

Chairman of Session on Interferon and Viral Inhibition at the International Congress of Virology in Budapest, 1971.

Publications:

Levy, H. B., and Riley, F.: The effect of polyinosinic.polycytidylic acid on tumor metabolism. Proc. Soc. Exp. Biol. Med. 135: 141-145, 1970.

DeVita, V. T., Cannellos, G. P., Carbone, P. P., Baron, S., Levy, H., and Grainick, H.: Clinical trails with interferon inducer polyinosinic.polycytidylic acid. Proc. Amer. Assoc. Cancer Res. 11: 21, 1970.

Weinstein, A. J., Gazdar, A. F., Sims, H., and Levy, H. B.: Lack of correlation between interferon induction and antitumor effect. Nature New Biology 231: 53-56, 1971.

Hill, D. A., Baron, S., Levy, H. B., Bellanti, J., Buckler, C. E., Cannellos, G., Carbone, P., Chanock, R. M., DeVita, V., Guggenheim, M. A., Homan, E., Kapikian, A. Z., Kirschstein, R. L., Mills, J., Perkins, J. C., VanKirk, J. E., and Worthington, M.: Clinical studies of induction of interferon by polyinosinic.polycytidylic acid. In Pollard, M. (Ed.): From Molecules to Man. New York, N. Y., Academic Press, 1971, pp. 197-217.

Dianzani, F., Baron, S., Buckler, C. E., and Levy, H. B.: Mechanisms of DEAE-dextran Enhancement of polynucleotide induction of interferon. Proc. Soc. Exp. Biol. Med. 136: 1111-1114, 1971.

Levy, H. B.: Interferon and interferon inducers in the treatment of neoplastic diseases. In Brodsky, I., and Kahn, S. B. (Eds.): Cancer Chemotherapy II. New York, N. Y., Grune & Stratton, 1972, pp. 53-61.

Levy, H. B., and Merigan, T. C.: Preparation and mode of action of interferon. In Last, J. A., and Laskin, A. I. (Eds.): Protein Biosynthesis in Nonbacterial Systems. New York, N. Y., Marcel Dekker, Inc., 1972, pp. 295-315.

Adamson, R. H., Levy, H. B., and Baron, S.: The interferon system. In Rubin, A. A. (Ed.): Search for New Drugs. New York, N. Y., Marcel Dekker, Inc., 1972, pp. 292-311.

Margolis, S. A., Oie, H., and Levy, H. B.: The effect of interferon, interferon inducers or interferon induced viral resistance on subsequent interferon production. J. Gen. Virol., 1972, in press.

Serial No. NIAID-71-G
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure and Replication of Viruses

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigators: Dr. Jerome F. Hruska
Mr. Wendell A. Daniel

Cooperating Units: Dr. K. K. Takemoto, LVD, NIAID, 71-J
Dr. Louis S. Diamond, LPD, NIAID, 122-G1
Dr. H. G. duBuy, LVD, NIAID, 71-I
Dr. George C. Lavelle, LVD, NIAID
Dr. S. Nomura, VLL, NCI
Dr. R. Bassin, VLL, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	2 3/12			2 3/12
Professional	2			2
Other	3/12			3/12

Project Description:

Objectives

The major emphasis of this project falls into two areas: (1) Slow viruses. Sheep pneumonitis virus morphogenesis is being studied in a tissue culture system. A collaborative study with Dr. Lavelle is aimed at identifying the scrapie agent. (2) The study of biological and chemical properties of ameba viruses and possible relationships to human disease.

Additional collaborative studies involve clones of murine sarcoma transformed mouse cells and their spontaneous revertents ("nontransformed") and rerevertents ("retransformed").

Methods Employed

These studies involve high resolution electron microscopy of viruses and sections of infected and normal cells employing a variety of stains. A variety of chemical and physical techniques are utilized in the purification and study of the biological and chemical properties of viruses.

Major Findings

(1) Virus isolates. To date 7 strains of axenized E. histolytica have been examined for viruses. All have been found to contain agents which lyse one or more other strains of amebae. Each donor amebal strain is resistant to its indigenous virus. ABRM is the only strain naturally carrying the filamentous virus. ABRM also contains a polyhedral agent as do the other 6 amebal strains. Host range specificity suggests that there are at least 2 polyhedral agents and possibly more.

(2) We have developed a relatively efficient assay system and investigated many properties of the polyhedral ameba virus, (HB-301)-->HK-9:

a) Growth curve: With a multiplicity of about 0.1 the HB-301 virus cultivated in HK-9 amebae replicates to a maximum titer (10^6 ID_{50}/ml) in about 48 hours.

b) Thermal inactivation: The virus is inactivated at 37C at the rate of about 0.8 log/24 hours in TPS-1 medium (amebal growth medium containing 10% bovine serum). It is inactivated much more rapidly at 37C in 0.1M tris buffer (pH 7.6).

The virus remains fully active when stored in medium at 4C for 20 days.

c) Freezing: The virus is inactivated when frozen in medium at -20C about 1 log with each of the first and second freezing-thawing, and to a lesser extent upon subsequent freezing and thawing. At -78.5C the virus is much more rapidly inactivated by repeated freezing and thawing.

d) pH Stability: The virus is relatively stable only within the pH range 6.5 to 8.0.

e) Neutralization by antibody: Antisera produced in rabbits inactivate 10 ID_{50} at a 1:1000 serum dilution and at least 10^4 ID_{50} at 1:150 dilution.

f) Virus inactivation: More than 99% of virus inactivation accompanies the following treatments: sonication, ethyl ether, chloroform, EDTA (10^{-2}M), SDS (1%), Alrowet (0.1%). Partial inactivation is observed with DOC (1%), Non Idet (1%), Tween 20 (0.1%). The virus is unaffected by trypsin (0.1%), Tris buffer (0.1M, pH7.6), hyaluronidase (100Y/ml), Triton X(0.1%), RDE(1:400), Non Idet (0.1%) and phosphalipase A2.

(3) Attempts to purify the virus have been hampered by the fact that the polyhedral ameba virus is very tightly bound to various membranes formed upon lysis of amebae. To date we have employed a variety of physical and chemical methods to free the virus, all unsuccessful.

(4) Attempts have been made to free HB-301 amebae of indigenous virus for two reasons. First is the need for a virus-free amebae strain, and second is that the ability to free a culture of virus would suggest that the axenized amebae virus systems were carrier cultures rather than lysogenic. Normal HB-301 amebae cultures contain $10^{2.5}$ ID_{50} virus as detected by grinding the amebae and assaying in HK-9 amebae. During 35 consecutive passages of HB-301 amebae in the presence of HB-301 virus antiserum, cultures remained negative for virus. However, seven select passages of this series were also cultured without antiserum and within 4-6 passages virus invariably reappeared to the original titer of $10^{2.5}$ ID_{50}/ml. Thus, sofar, we have been unable to permanently free an axenized amebae strain of virus by culturing in the presence of antiserum. These observations are compatible with, but not conclusive proof of, this being a lysogenic system.

(4) Collaborative studies (NCI) on murine leukemia and sarcoma viruses have revealed spontaneous reversion of MLV transformed cell clones to morphologically untransformed cells which nonetheless maintain some sarcoma gene potential. C particles are inducible by BUDR, but they are noninfectious and cannot be rescued by MLV. A manuscript has been prepared and is ready for submission.

Significance to Biomedical Research and the Program of the Institute

These studies are intended to contribute to our knowledge of the processes by which both RNA and DNA viruses replicate. The

ultimate goal, which is finding means of interrupting the viral replication cycle, will undoubtedly require approaches involving multiple disciplines, including such electron microscopic studies.

(1) The possible medical significance of protozoal viruses is being investigated.

(2) Continued basic information on any of the slow viruses may prove valuable in the study of a number of chronic diseases in man and animals.

(3) Studies on the mechanisms of murine sarcoma transformation and reversion of cells may provide clues as to basic processes in human malignancies.

Proposed Course of the Project

(1) Continued effort is being expended to elucidate virus replication by means of electron microscopy coupled with various collaborative studies employing other methods. The major effort will be on slow viruses such as sheep pneumonitis, visna and scrapie.

(2) Continued intensive study of all phases of the new ameba viruses is planned in collaboration with LPD. It is also planned to explore the possibility that other human pathogens may have indigenous viruses. Serological screening of patients with amebiasis to determine if they have had an antibody response to ameba viruses is in progress. Likewise animal models of hepatic disease will be treated with appropriate amebal viruses to attempt to alter the course of the disease. Attempts are in progress to cultivate amebal viruses in a variety of vertebrate tissue culture-systems.

Publications:

Takemoto, K. K., Mattern, C. F. T., and Murakami, W. T.: The papova virus group. In Maramarosch, K., and Kurstak, E. (Eds.): Comparative Virology. New York, Academic Press, 1971, pp. 81-104.

Ellison, J. S., Mattern, C. F. T., and Daniel, W. A.: Structural changes in Clostridium botulinum type E following treatment with boticin S51. J. Bacter. 108: 526-534, 1971.

Diamond, L. S., Mattern, C. F. T., and Bartgis, I. L.: Viruses of Entamoeba histolytica. I. Identification of transmissible virus-like agents. J. Virol. 9: 326-341, 1972.

Mattern, C. F. T., Diamond, L. S., and Daniel, W. A.: Viruses of Entamoeba histolytica. II. Morphogenesis of the polyhedral particle (ABRM2-->HK-9)-->HB-301 and the filamentous agent (ABRM)2-->HK-9. J. Virol. 9: 342-358, 1972.

Serial No. NIAID-71-G1
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biological Ultrastructure

Previous Serial Number: SAME

Principal Investigator: Dr. C. F. T. Mattern

Other Investigator: Mr. Wendell A. Daniel

Cooperating Units: Dr. Bronislaw M. Honigberg, Dept. Zoology, University of Massachusetts
Dr. Robert I. Henkin, Clinical Endocrinology Branch, NHLI
Dr. H. G. duBuy, LVD, NIAID, 71-1
Dr. Ruth Pertel, LPD, NIAID, 122-J

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	1 3/12		8/12	1 11/12
Professional	1		8/12	1 8/12
Other	3/12			3/12

Project Description:

Objectives

To study the ultrastructure of protozoa, metazoa, and vertebrates with particular emphasis on the correlation of ultrastructure and function. Organisms and organs under study are selected both to study phylogenetic kinships and certain details of embryogenesis.

Methods Employed

High resolution electron microscopic observation of sectioned cells and organisms is emphasized. In addition, organs, cells and isolated cellular organelles are investigated for ultrastructural detail by additional techniques including negative and positive stain, light microscopy with conventional staining, fluorescent antibody staining and dye binding.

Major Findings

(1) Continued collaborative studies on the ultrastructure of trichomonads have included Trichomitus batrachorum. A detailed study of its mastigont complex has led to further clarification of the evolutionary kinships of these protozoa.

T. batrachorum shares in common with Trichomonadinae many fine structural characteristics, particularly its undulating membrane, and periodic marginal lamella. On the other hand certain structures, the costal base and comb-like organelle, resemble those of the Monocercomonas and the origin of the marginal lamella is remarkably similar to that of Hypotrichomonas. Further, T. batrachorum resembles the Tritrichomonadinae in respect to both its comb-like structure and type A costa not found in Hypotrichomonas or Trichomonadinae. These observations support the evolutionary sequence, Monocercomonas-->Hypotrichomonas-->Trichomitus-->Trichomonadinae and Tritrichomonadinae.

(2) Studies on pseudocyst formation of T. batrachorum have revealed a unique situation in which the recurrent flagellum becomes internalized and the flagellar microtubules dissociate. This is accompanied by an accumulation of a large number of fine filaments presumed to represent another form of microtubular protein.

(3) Investigations on the ultrastructure of the taste apparatus have led to a comparative study of human, insect and nematode contact chemoreceptors. In the latter two the chemoreceptors consist of modified cilia. In collaborative studies attempts are being made to correlate the ultrastructural findings and physiological mechanisms involved in chemoreceptors.

Continued studies of normal and pathological taste buds in man have revealed ultrastructural changes associated with Sjogrens syndrome.

Significance to Biomedical Research and the Program of the Institute

Due to the variety of functions of cilia, flagella and kinetosomes, these continued studies remain of great potential importance. Some potential areas of importance are in birth control either by control of flagellar activity of sperm or by alteration of the normal ciliary activity in the oviduct as well as in the control of malignant disease by limiting the centriole's activity in producing spindle microtubules. The latter approach has been employed clinically through the use of vincristine in leukemia therapy.

Proposed Course of the Project

These comparative ultrastructural studies of the anatomy of protozoa, metazoa, vertebrate cells and organs will be continued. They will be correlated with physiological studies of vertebrate and invertebrate organs.

Publications:

Mattern, C. F. T.: The development of muscle; phylogenetic and embryological considerations. In Laki, K. (Ed.): Contractile Proteins and Muscle Contraction. New York, Dekker, 1971, pp. 355-412.

Henkin, R. I., Schechter, P. J., Hoye, R., and Mattern, C. F. T.: Idiopathic hypogusia with dysgusia and dysosmia; A new syndrome. J. Amer. Med. Assoc. 217: 434-440, 1971.

Mattern, C. F. T., and Honigberg, B. M.: Observations on the undulatory motion of the costa of Trichomitopsis termopsidis. Trans. Amer. Microsc. Soc. 90: 309-313, 1971.

Honigberg, B. M., Mattern, C. F. T., and Daniel, W. A.: The fine structure of the mastigont system of Tritrichomonas foetus (Riedmuller). J. Protozool. 18: 183-198, 1971.

Daniel, W. A., Mattern, C. F. T., and Honigberg, B. M.: Fine structure of the mastigont system in Tritrichomonas muris (Grassi). J. Protozool.18: 575-586, 1971.

Henkin, R. I., Talal, N., Larson, A. L., and Mattern, C. F. T.: Abnormalities of taste and smell in Sjogren's syndrome. Ann. Int. Med. 76: 375-383, 1972.

Mattern, C. F. T., Honigberg, B. M., and Daniel, W. A.: Structure of Monoceromonas Sp. as revealed by electron microscopy. J. Protozool. 19: 265-274, 1972.

Honigberg, B. M., Daniel, W. A., and Mattern, C. F. T.: Fine structure of Trichomitus batrachorum (Perty). J. Protozool., in press, 1972.

Serial No. NIAID-71-H
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host defenses against viral infection: Interferon and Immunity

Previous Serial Number: SAME

Principal Investigator: Dr. Samuel Baron

Other Investigators: Dr. Michael Worthington, LVD, NIAID
Charles E. Buckler, LVD, NIAID

Cooperating Units: Dr. F. Dianzani, Guest Worker, LVD, NIAID (71-H)
Dr. Ronald Clark, DBS, NIH
Dr. Herbert F. Hasenclever, LM, NIAID, (79-B-1)
Dr. Herman duBuy, LVD, NIAID, (71-I)
Dr. Alfred D. Steinberg, NIAMD, NIH
Dr. Robert Herman, LPD, NIAID, (125-Q)
Dr. Mercedes Weissenbacher, New York Medical College

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	4		2/12	4 2/12
Professional	3		2/12	3 2/12
Other	1			1

Contract Support:

Contract Number	Project Officer	Contractor	Contractor's Project Director	Annual Funding Level
NIH 71-674	Dr. S. Baron	North American Biologicals, Inc.	Dr. R.R.Rafajko	$44,334
NIH 72-C-64466	"	"	"	$31,666

Project Description:

Objectives

Host defenses (a) during infections and (b) as possible control measures for viral infections were studied using several different approaches.

1. Factors affecting polynucleotide induction of interferon.

2. Effect of immunity and immunosuppression during infections.

3. The effect of a polynucleotide inducer of interferon during protozoa and fungal infections.

Methods Employed

A variety of cell cultures were treated with polyinosinic.polycytidylic (poly I.poly C) acid (a synthetic RNA polynucleotide inducer of interferon) in the presence and absence of substances which interact with cells and polyinosinic.polycytidylic acid. Observations were made for enhancement of interferon induction under these conditions and for clues to the mechanism of action of such enhancement. In other experiments mice were immunized with polynucleotides and the effects of the resulting antibody on the ability of poly I.poly C to induce interferon in vivo and in vitro were determined. Other mice were induced to produce interferon and were then infected with *T. congolense* or with one or several fungi. The course of infection and disease in the experimental animals was compared to those of control animals. In other studies mice were treated with the immunosuppressive drugs cyclophosphamide, azathioprine, or 6-mercaptopurine and then either stimulated to produce interferon with polyinosinic.polycytidylic acid or infected with lactic dehydrogenase virus or scrapie virus. The effect of the

immunosuppressive agents on interferon production or the course of infections was followed.

Major Findings

1. Factors affecting polynucleotide induction of interferon. DEAE-dextran strongly enhances the production of interferon by polynucleotides. Two possible mechanisms underlying the enhancing effect are (a) protection of the polynucleotide inducer against extracellular ribonuclease by DEAE-dextran and (b) a cell mediated effect of DEAE-dextran which may be related to increased permeability for the large polynucleotide molecules. To help determine the relative importance of these factors we examined the enhancing effect of DEAE-dextran on a ribonuclease resistant form of poly I.poly C. It was found that the induction of interferon by the ribonuclease resistant complex was potentiated by the addition of DEAE-dextran to cell cultures. This indicates that protection of poly I.poly C against enzymatic degradation is not the sole mechanism by which interferon production can be enhanced by DEAE-dextran. It also implies that DEAE-dextran exerts a large part of its enhancement by an effect on cells.

Because of the potential beneficial effects of interferon induction on human ocular infection by herpes simplex virus, a study was undertaken to evaluate the enhancing effect by neomycin on the interferon stimulating effect of poly I.poly C on rabbit ocular tissues in culture. The induction of the interferon mechanism in rabbit corneal, conjunctival, iris and kidney tissue cultures with poly I.poly C protected against infections with herpes simplex virus. The degree of protection was inversely proportional to the virus challenge dose and was substantially enhanced by the addition of neomycin. The enhancing effect of neomycin was most pronounced when small doses of poly I.poly C were used. These findings indicate that polybasic substances like neomycin act with polynucleotide inducers of interferon in ocular cell culture to enhance submaximal induction of interferon.

The relationship in mice between immune status to poly I.poly C and interferon induction by poly I.poly C was studied in vivo and in vitro. Circulating antibody to poly I.poly C reduced the in vivo interferon response to poly I.poly C. A similar but less profound inhibitory effect of specific antibody upon interferon induction was demonstrated in vitro. The findings imply that antibody-poly I.poly C immune complexes are less potent interferon inducers than poly I.poly C without antibody. Immunological tolerance to poly I.poly C did not alter interferon

induction by poly I.poly C indicating dissociation between immunological status and interferon induction with this synthetic RNA in vivo.

2. Effects of immunity and immunosuppression during infection. Although scrapie infection has been studied for many years, its pathogenesis is still poorly understood. It has not been possible to demonstrate any immune response to the scrapie agent and it is not known if immune mechanisms play any role in defense against scrapie infection. Because of this uncertainty experiments were undertaken to determine the effect of long term immunosuppression with cyclophosphamide on the outcome of scrapie infection in the mouse. Immunosuppressed and control mice developed clinical signs of scrapie disease after about six and a half months with no difference in incubation time or development of disease between the groups. There was also no difference between the times of death for each group. The results suggest that in scrapie infection in the mouse the immune system does not play a major role in the development of, or protection against, the observed diseases.

In a similar study cyclophosphamide was used to study the effect of immunosuppression on the degree of chronic lactic dehydrogenase virus viremia in mice. Weekly injections of cyclophosphamide caused an increase of LDV viremia which occurred at about the same time that the formation of antibody could be demonstrated in the serum of control mice. In the cyclophosphamide treated mice neither neutralizing antibody or "sensitized" virus were produced. The increased viremia in the immunosuppressed mice during the later stages of infection indicates that the immune response plays an important role in control of the chronic viremia. The quantitative correlation between the decrease in neutralizing antibody and the increase in viremia in immunosuppressed mice suggests that antibody rather than cellular immunity may be the more important immune factor in the control of this chronic viremia. This study will be described in detail in the report by Dr. H. duBuy, LVD, NIAID.

A study was performed to determine whether several clinically important immunosuppressive agents influenced interferon production and the protective effect of an interferon inducer in mice. Large doses of cyclophosphamide, 6-mercaptopurine and azathioprine had no effect on the stimulation of interferon by poly I.poly C or Newcastle disease virus in mice. Treatment with poly I.poly C was effective in protecting cyclophosphamide-treated mice from fatal systemic vaccinia infection even when poly I.poly C treatment was begun 4 or 5 days after virus injection and at a time when definite tail

lesions had already developed. Thus under the conditions of these experiments the three immunosuppressive agents did not suppress the inteferon mechanism.

3. Effect of a polynucleotide inducer of interferon during protozoa and fungal infections. The administration of an interferon stimulator, poly I.poly C increased the severity of both experimental Candida albicans and Coccidioides immitis infections in mice, as evidenced by earlier time of death and higher final mortality. In addition, kidney tissue levels of C. albicans at three and five days after infection were found to be about 40 to 300-fold higher in poly I.poly C treated mice than in control mice. Experimental Cryptococcus neoformans infection, however, was not significantly affected by poly I.poly C treatment.

The synthetic polynucleotides, poly I.poly C and poly A.poly U were tested for protective effect in mice infected with T. congolense. The protective effects of poly I.poly C and poly A.poly U varied independently of each other as assayed by the mean parasitemias and cumulative mortalities of infected mice. Poly I.poly C resistant and poly I.poly C susceptible variants of T. congolense were isolated. The protective effects of the polynucleotides were probably due to their immunologic enhancing capacity and not due to interferon. This interpretation comes from the observations that injections of Newcastle disease virus, a strong inducer of interferon in mice, did not protect against T. congolense infections. Also the protective effects of poly I.poly C in T. congolense infected mice were reversed by treatment of with the immunosuppressive agent, cyclophosphamide. These findings emphasize that interferon inducers may have effects due to non-interferon mechanisms. This study will be described in detail in Dr. Herman's report (LPD, NIAID).

Significance to Biomedical Research and the Program of the Institute

Studies of the factors affecting the polynucleotide induction of interferon are important because to date polynucleotides are relatively poor inducers of interferon in man as compared with the mouse or the rabbit. With the knowledge that man can produce much interferon in reponse to viral infections, information leading to control of the induction process by polynucleotides offers the possibility of increasing the interferon response to polynucleotides and therefore perhaps to the eventual control of certain viral infections of man.

Studies of the effects of immunosuppression on viral infections are important because during immunosuppression of man (for treatment of malignant disease or prevention of transplant organ rejection) enhancement of certain viral infections can occur. The present studies suggest that the interferon mechanism is intact during immunosuppression and therefore interferon inducers could be used in these clinical situations to help control any enhanced viral infections. Furthermore the findings help delineate the relative roles of antibody, cellular immunity and interferon during viral infection. Specifically the immune response during scrapie infection seems to play little role in the pathogenesis of disease whereas the immune response during lactic dehydrogenase virus infection of mice plays a partial role.

Studies of the effects of polynucleotides during protozoal and fungal infections are significant because they demonstrate that the polynucleotides can affect certain protozoal infections via the polynucleotide enhancement of the immune response. Also the enhancement of several fungal infections by polynucleotides points up a possible drawback to the application of polynucleotide inducers for control of human viral infections.

Proposed Course of the Project

Studies of the factors affecting polynucleotide induction of nterferon will be continued to help better understand the mechanism of induction of interferon. In this way it is hoped that induction of high levels of interferon in man can eventually be achieved. Studies of the effect of immunosuppressive agents during viral infection will be extended to several virus-host systems to continue to dissect the relative roles of antibody, cellular immunity and interferon during these infections. Model infections employed will utilize infections of different target organs and utilize viruses which spread to the target organs via various routes. The studies of the effect of polynucleotides during infections will continue, but with the emphasis on viral infections, with the hope of using the information gained in the various other areas of research to achieve control of viral infections.

Honors and Awards:

Associate Editor, American Journal of Epidemiology

Associate Editor, Infection and Immunity

Associate Editor, International Journal of Cancer

Member of Committees for American Association of Immunologists, and American Society for Microbiology

Elected outstanding alumnus member of New York University Chapter of AOA

Invited lectures at Universities, Research Institutes, and Symposia

Publications:

Weissenbacher, M., Galin, M. A., Chowchuvech, E., Schachter, N., and Baron, S.: Protection by polyinosinic-polycytidylic acid complex of rabbit eye tissue cultures infected with herpes simplex virus. Invest. Ophthal. 9: 857-864, 1970.

Dianzani, F., Baron, S., Buckler, C. E., and Levy, H. B.: Mechanisms of DEAE-dextran enhancement of polynucleotide induction of interferon. Proc. Soc. Exp. Biol. Med. 136: 1111-1114, 1971.

Worthington, M., and Clark, R.: Lack of effect of immunosuppression on scrapie infection in mice. J. Gen. Virol. 13: 349-351, 1971.

duBuy, H. G., Worthington, M., and Johnson, M. L.: Effect of an immunosuppressive agent, cyclophosphamide, on chronic Lactic Dehydrogenase virus viremia of mice. Infect. and Immun. 4: 720-724, 1971.

Steinberg, A. D., Baron, S., Uhlendorf, C., and Talal, N.: Depression of the interferon response to polyinosinic.polycytidylic acid by specific antibody. Proc. Soc. Exp. Biol. Med. 137: 558-561, 1971.

Herman, R., and Baron, S.: Immunologic-mediated protection of Trypanosoma congolense infected mice by polyribonucleotides. J. Protozool. 18: 661-666, 1971.

Worthington, M., and Baron, S.: Effectiveness of an interferon stimulator in immunosuppressed mice. Proc. Soc. Exp. Biol. Med. 136: 349-353, 1971.

Hill, D. A., Perkins, J. C., Worthington, M., Kapikian, A. Z., Chanock, R. M., and Baron, S.: Preliminary study of the effect of poly I.poly C on experimental rhinovirus infection in volunteers. In Sanders, M., and Schaeffer, M. (Eds.): Viruses Affecting Man and Animals. St. Louis, Mo., Warren H. Green, Inc., 1971, pp. 404-415.

Baron, S.: Mechanism of stimulation of interferon production by viruses and RNAs and its application. In Sanders, M., and Schaeffer, M. (Eds.): Viruses Affecting Man and Animals. St. Louis, Mo., Warren H. Green, Inc., 1971, pp. 89-99.

Baron, S., duBuy, H., Buckler, C. E., Johnson, M., and Worthington, M.: Factors affecting the interferon response of mice to polynucleotides. In Beers, R. F., Jr., and Braun, W. (Eds.): Biological Effects of Polynucleotides. New York, N. Y., Springer-Verlag, 1971, pp. 45-54.

Hill, D. A., Baron, S., Levy, H. B., Bellanti, J., Buckler, C. E., Cannellos, G., Carbone, P., Chanock, R. M., DeVita, V., Guggenheim, M. A., Homan, E., Kapikian, A. Z., Kirschstein, R. L., Mills, J., Perkins, J. C., VanKirk, J. E., and Worthington, M.: Clinical studies of induction of interferon by polyinosinic.polycytidylic acid. In Pollard, M. (Ed.): From Molecules to Man. New York, N. Y., Academic Press, 1971, pp. 197-217.

Worthington, M., and Hasenclever, H. F.: Effect of an interferon stimulator, polyinosinic:polycytidylic acid, on experimental fungus infections. Infect. & Immun. 5: 199-202, 1972.

Adamson, R. H., Levy, H. B., and Baron, S.: The interferon system. In Rubin, A. A. (Ed.): Search for New Drugs. New York, N. Y., Marcel Dekker, Inc., 1972, pp. 291-316.

Baron, S.: Interferon as a general antiviral agent. Science Teacher 39: 43-48, 1972.

Serial No. NIAID-71-I
1. Laboratory of Viral Diseases
2. Molecular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The interaction between latent and slow viruses, especially the Lactic Dehydrogenase Virus (LDV) and the subcellular elements of the host cell (mouse macrophages for LDV). Use of the LDV-modified macrophages model for the study of role of macrophages in the defense mechanisms of the host.

Previous Serial Number: SAME

Principal Investigator: Dr. Herman G. duBuy

Other Investigators: None

Cooperating Units: Dr. C. F. T. Mattern, LVD, NIAID, 71-G
Dr. Samuel Baron, LVD, NIAID, 71-H

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	2			2
Professional	1			1
Other	1			1

Project Description:

Objectives

Study of the factors, both in vivo and in vitro, which determine whether a virus kills the host, becomes chronic, or is eliminated, and of factors which modify the outcome of the virus infection: Interferon and its inducers, humoral antibody, and cellular defense factors, especially those of macrophages, the target cells of LDV.

Methods Employed

The murine Lactic Dehydrogenase virus is used as a model because it is an RNA membrane virus which multiplies exclusively in mouse macrophages, leading to a chronic viremia without any apparent morbidity of the host. Subprojects have been developed on the basis of results obtained with the mouse macrophage-LDV model, which allows the determination of changes in macrophage functions (clearance of serum enzymes, reaction to interferon and its inducers, rate of LDV-replication in macrophages in vitro, etc.)

Major Findings

(1) The sensitivity of LDV to interferon, as compared with that of two other viruses was determined, by using peritoneal mouse macrophage cultures (in collaboration with Mrs. C. Uhlendorf) for interferon assay. This was done by adaptation of the standard procedure, cultivating the macrophages in test tubes held vertically in a spring rack and covered loosely with a single metal lid. The sensitivity to interferon, in decreasing order, was: vesicular stomatitis virus, Sendai virus and LDV.

(2) The prolonged treatment of mice with chronic LDV viremia with the immunosuppressive drug cyclophosphamide not only led to the absence of neutralizing antibody against LDV, but also to the absence of sensitized, antibody-bound but infectious virus, resulting in an increased viremic titer. Interestingly, the effect lasted weeks after cessation of the treatment.

(3) In the study of various regimens of interferon applied to mice to eliminate the LDV viremia, it was found that neither i.v. nor i.p. injections of large amounts of interferon eliminated the viremia, but caused a reduction.

(4) The study of the effect of different routes of application of interferon or its inducers led to a subproject, in which the effect of prolonged local or distal therapeutic treatment with poly I.poly C on the survival time of mice inoculated with Ehrlich ascites carcinoma was studied. It was found that the better the contact of the individual cancer cells with the poly I.poly C, the more effective the treatment, depending on the form of the tumor and the route of poly I.poly C injection.

Significance to Biomedical Research and the Program of the Institute

The study of the chronic viremia caused by LDV contributes to the understanding of other chronic viremias and latent infections. Because of the involvement specifically of macrophages in the macrophage-LDV model, it may show a possible role of macrophages in resistance to chronic viral diseases and in tumor immunity.

Proposed Course of the Project

Continuation of the studies to eliminate chronic LDV viremia by using interferon inducers with differing properties, in conjunction with physical or chemical factors that change macrophage function, either in vivo or in vitro, in order to prevent the replication of LDV in the macrophages. For example, the isolation of an m-RNA for the antiviral state is in progress, to be tested in the LDV-macrophage system.

Publications:

duBuy, H. G., Worthington, M., and Johnson, M. L.: Effect of an immunosuppressive agent, cyclophosphamide, on chronic Lactic Dehydrogenase virus viremia of mice. Infect. and Immun. 4: 720-724, 1971.

duBuy, H. G.: Effect of local or distal therapeutic treatment with polyinosinic.polycytidylic acid on survival of mice with ehrlich carcinoma. J. Natl. Cancer Inst., in press, 1972.

Baron, S., duBuy, H., Buckler, C. E., Johnson, M. L., and Worthington, M.: Factors affecting the interferon response of mice to polynucleotides. Beers, R. F., Jr., and Braun, W. (Eds.): Biological Effects of Polynucleotides. New York, N. Y., Springer-Verlag, 1971, pp. 45-54.

Serial No. NIAID-71-J
1. Laboratory of Viral Diseases
2. Cellular Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Oncogenic and "Slow" Viruses

Previous Serial Number: SAME

Principal Investigator: Dr. Kenneth K. Takemoto

Other Investigators: Dr. M. A. Martin, LBV, NIAID, (80-A)
Dr. L. Gelb, LBV, NIAID, (80-A)
Dr. C. Garon, LBV, NIAID, (80-A)
Dr. J. Coe, RML, NIAID, (175)
Dr. K. Chang, LCB, NCI, (55-50)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total	2			2
Professional	1			1
Other	1			1

Project Description:

Objectives

(1) To study tumor viruses and tumor cells from a broad standpoint in order to elucidate mechanisms involved in viral carcinogenesis; (2) To characterize heretofore unclassified viruses which cause slow and chronic infections and examine their relationship to oncogenic viruses.

Methods Employed

Standard cell culture and virologic methods are used.

Major Findings

Last year, we reported our findings on the remarkable similarities of the morphological, biochemical and biological properties of visna and progressive pneumonia viruses, the causative agents of slow neurologic and pulmonary diseases of sheep, to those of the oncogenic RNA viruses. The common properties shared by these two groups of viruses include the following:

1. Presence of RNA-dependent DNA polymerase
2. Similar virion size and structure
3. Requirement for DNA synthesis for replication
4. Single large 70S RNA as its genome
5. Ability to transform cells

In the current year, these studies have been continued in efforts to establish a firm basis for a tentative taxonomic classification of these viruses within the RNA tumor virus group. Additional findings during the past year lend further support for this conclusion. These include the following:

A. Ultrastructural studies on the visna virion. Employing procedures which differed from those previously used, highly purified visna virions were prepared for electron microscopy. In collaboration with Dr. Claude Garon (LBV), the visna virion was found to contain well-defined surface projections which appear to resemble those of the avian tumor viruses. The internal structure also appeared to be similar to those recently described for RNA tumor viruses, with suggestive helical arrangement.

B. Poly A regions in visna RNA. Recent studies with RNA tumor viruses have shown that the RNA from tumor viruses, unlike that from non-oncogenic RNA viruses, contain regions of polyadenylic acid (Poly A). All the RNA tumor viruses thus far examined (avian, murine, feline, and monkey) appear to have these poly A tracts, and therefore seem to be another basis for virus classification. In collaboration with Dr. David Gillespie (NICHD), similar tracts of poly A have been detected in the 70S RNA extracted from visna virus. The similarities of visna with the oncogenic RNA viruses has thus been extended to the composition of the RNA.

C. Effect of Rifamycin derivatives on visna replication. Rifamycin, Rifampicin, and various derivatives have been reported to inhibit the reverse transcriptase of RNA tumor viruses. Others have also reported that these compounds also inhibit focus-forming activity of RNA tumor viruses, presumably by inhibiting polymerase activity. When some of these compounds were tested against visna, an unexpected enhancement of virus growth was observed. Rifamycin SV, at concentrations as low as 3 ug/ml causes a 10-fold increase in virus yields, and a much more rapid cytopathic effect was observed. The mechanism involved in this enhancement is under current investigation. The effect appears to be highly specific for visna virus, since other viruses, including the simian foamy virus which also has reverse transcriptase activity, are completely unaffected by the drug.

This observation has enabled the development of a rapid 12-day plaque assay for visna, a virus which heretofore has been extremely difficult to assay.

D. Transformation by visna virus. Additional studies on transformation by visna have revealed the following:

1. Early passage mouse embryo cells are much more sensitive to transformation by visna, compared with continuous cell lines. Transformation occurs much sooner (by 10 days) and at higher efficiency.

2. Rat kidney cells (NRK cell line) can be transformed by visna.

3. Visna interferes with focus-formation by murine sarcoma virus. Mouse cells previously exposed to visna develop a resistance to MSV and the number of foci is approximately 10-fold less than untreated cells. The basis for this resistance is currently being investigated.

Proposed Course of the Project

Studies on the possible relationship of slow viral infections and oncogenic viruses will be continued. While visna will be the principal virus employed for these studies, work on other viruses with similar potential for causing either slow, chronic infections or with tumorigenic ability will be initiated. One of these is the recently described papovavirus from cases of progressive multifocal leucoencephalopathy; the other is the RNA-containing syncytium-forming (foamy) viruses which have reverse-transcriptase.

Significance to Biomedical Research and the Program of the Institute

Information obtained from the study of relationships between "slow" viruses and tumor viruses may provide insight into the mechanisms of the disease processes induced by these viruses. These investigations have revealed that viruses which have the potential to "integrate" viral genes in infected cells may play an important role in slow, chronic, or latent viral infections.

Honors and Awards:

1. Participated in meeting on multiple sclerosis sponsored by the Maryland Chapter, Multiple Sclerosis Society, Easton, Maryland, April, 1971.

2. Participated in conference on Biology of Cancer Cells, University of Colorado, Boulder, Colorado, July, 1971.

3. Participated in Multiple Sclerosis Conference sponsored by Reed Neurologic Center, UCLA and the Kroc Foundation, held at Santa Ynez, Calif., February, 1972.

4. Appointed Associate Editor, Journal of the National Cancer Institute.

5. Awarded the Public Health Service Commendation Medal.

Publications:

Takemoto, K. K., and Stone, L. B.: Transformation of murine cells by two "slow viruses" visna and progressive pneumonia. J. Virol. 77: 75, 1971.

Stone, L. B., Takemoto, K. K., and Martin, M. A.: Physical and biochemical properties of progressive pneumonia virus. J. Virol. 8: 573-578, 1971.

Rhim, J. S., Lengel, C. R., Takemoto, K. K., and Huebner, R. J.: Accelerated transformation by polyoma virus in rat embryo cells infected with Rauscher leukemia virus. Proc. Soc. Exp. Biol. Med. 138: 308-311, 1971.

Ting, C. C., Lavrin, D. H., Takemoto, K. K., Ting, R. C., and Herberman, R. B.: Expression of various tumor specific antigens in polyoma virus-induced tumors. Cancer Res. 32: 1-6, 1972.

Rhim, J. S., Huebner, R. J., Takemoto, K. K., and Gilden, R. J.: In vitro carcinogenesis studies: Dual effect of RNA tumor viruses, carcinogenic chemicals, and DNA viruses. 5th International Symposium Comparative Leukemia Research, Padova, Italy, 1971, in press.

Coe, J. E., and Takemoto, K. K.: Immune response in the hamster. VI. Antibody response in polyoma oncogenesis. J. Natl. Cancer Inst., 1972, in press.

Takemoto, K. K.: Visna and Progressive pneumonia viruses: recent findings on their similarities to oncogenic RNA viruses. Univ. Calif. Los Angeles Medical Forum. New York, Academic Press, 1971, in press.

Rhim, J. S., Lengel, C. R., Cho, H. Y., Takemoto, K. K., Turner, H. C., Huebner, R. J., and Gilden, R. V.: Expression of a new complement-fixing antigen reactive with murine sarcoma virus rat antiserum in rat cells transformed by polyoma virus. Nature 235: 188-189, 1972.

Sturman, L. S., and Takemoto, K. K.: Coronavirus infection of normal and transformed mouse cells. J. Virol., 1972, in press.

Oxman, M. N., Takemoto, K. K., and Eckhart, W.: Polyoma T-antigen synthesis by temperature sensitive mutants of polyoma virus. Virology, 1972, in press.

Scher, C. D., Takemoto, K. K., and Todaro, G. J.: The resistance of SV40 transformed 3T3 to supertransformation. Proc. Soc. Exp. Biol. Med. 136: 742-746, 1971.

LABORATORY OF MICROBIOLOGY

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PHS-NIH
Summary Statement
Laboratory of Microbiology, NIAID
July 1, 1971 through June 30, 1972

Introduction

Despite progressive small decrements in direct operating funds, LM has had no serious difficulties yet in performance of mission. A long-standing problem in common with other laboratories of NIAID is a need for better space; its lack slows work and also restricts our opportunities for utilization of post-doctoral fellows, from whom we have had many more good applications than we can fulfill. An increasing problem is that of replacement of personnel, coupled with cumulative frustrations of lowering employee morale because of stringency in promotion policy. We anticipate loss by retirement of 3 or 4 personnel by June 30, 1972. Mrs. Margaret DeLacy resigned in November 1971 and was replaced by Mr. Ronald D. Mayer in January 1972. No replacement for Dr. George Lones, in Mycology, has been obtained. Dr. Alan Glick, Staff Associate, will complete duty in June. We regretfully report the death in March of Miss Katherine Steele, secretary to the Mycology Section for over 14 years. New personnel include Dr. C. Lowell Parsons, Staff Associate, who replaced Dr. Richard Getnick in July 1971; and Dr. Unnur Petursdottir, Guest Worker from Iceland, who began work in February.

The principal mission of the Laboratory of Microbiology is construed to be the advancement of basic knowledge of prokaryotic microorganisms. Organisms studied include bacteria, fungi, mycoplasma, and bacterial viruses. Areas of research include genetics, metabolism, serology and immunology, taxonomy, ultrastructure, biochemistry, and all interrelations thereof. Particular emphasis is on structure-function relations of such isolatable microorganismal components as cell wall, cell membrane, organelles such as mesosomes, the DNA genetic apparatus; and chemical entities such as structural proteins, enzymes, and other biologically active molecules.

Bacterial Structure and Function Section

Effect of CO_2 on Bacterial Growth

In extension of prior studies on CO_2/bicarbonate requirements for Hydrogenomonas eutropha (an autotrophic aerobe), two different bacteria were studied. In a defined simple medium, Escherichia coli (a facultative heterotrophic aerobe) responded to CO_2 but not to bicarbonate concentration. A heterotrophic anaerobe, (Group H streptococcus, strain Challis), in a complex medium, appeared to require both CO_2 and bicarbonate--for growth as well as for production of competence factor and subsequent development of genetic competence. Neither organism initiated growth unless CO_2 was provided. All three bacteria can be grown without a lag period, even from old stationary phase cultures, if the proper CO_2/bicarbonate concentration is provided. (Repaske)

Protein, Glycoprotein, and Lipid Differences between Bacterial Membranes and Mesosomes

Mesosomes from a Group A streptococcus continued to show more protein bands than membranes on gel electrophoresis--regardless of whether the material

was delipidated; extracted with phenol-acetic, Triton-X or sodium dodecyl sulfate mixtures; or run on acid or neutral gels. By the different methods, however, the total number of bands varied from 20 to 34 for membranes, and 17 to 27 for mesosomes, respectively. (Schade) Mesosomes and membranes isolated from Staphylococcus aureus were extracted with sodium dodecyl sulfate and electrophoresed in gels containing this substance. Both showed 26 protein bands, whether or not previously delipidated, and no qualitative differences were seen. Membranes contained more high molecular weight proteins, while mesosomes had a higher concentration of those of low molecular weight. Other techniques extracted a presumptive glycoprotein and a glycophospho-lipid--both of which appear predominantly or exclusively, respectively, in the mesosomes. (Theodore) The precise natures and functions or of the proteins, or of the newly-isolated compounds, are not yet known--but the demonstration of distinct differences is encouraging in these efforts to show a distinctive function for mesosomes.

Cell Wall Structure, Autolysin, and Growth in Staphylococcus

Bacterial size and shape are obviously determined by the configuration and rigidity of the cell wall, but control of wall synthesis is poorly understood. To approach this problem, we have previously examined, ultrastructurally, mutants of bacilli which show abnormalities of cell wall ultrastructure, size, shape, and division. These have been temperature-conditional mutants showing absence of wall teichoic acid and of autolytic activity at the restrictive temperature. Recently, a non-conditional, teichoic acid--and autolysis-deficient mutant (tar-1) of Staphylococcus aureus H (str) was studied under a variety of conditions. The mutant grows in clusters of randomly-divided but non-separating large irregular cells with thickened and non-trilaminar walls--especially under unbalanced growth conditions (stationary phase cells, or growth at elevated temperature), which also cause increased cell wall density and thickening in both parent and mutant. Morphology and ultrastructure that is "normal" for both (i.e., characteristic of log phase growth) is restored or retained by 1 M NaCl added to the medium. In this condition, visible "loosening" of wall structure is seen by electron microscopy. Furthermore, molar NaCl added to cells in a "non-growth" buffer causes gross lysis of cells--especially those of the mutant--and wall substance can be seen to disintegrate progressively. These results suggest the participation of teichoic acid and autolysis in maintenance of wall structure, cell size, shape, and division in this staphylococcus. However, the existence of similar morphological mutants of bacilli with normal teichoic acid and autolytic activity, of normally-shaped bacilli lacking wall teichoic acid, makes any universal interpretations impossible at present. (Cole, Popkin; Collaborators Chatterjee, Young)

Intergroup Lysis and Transduction by Streptococcal Bacteriophages

In extension of previous studies, and in continuing refutation of the concept of serogroup specificity, bacteriophages from streptococci of Groups A, E, and G were found to lyse (by the surface plate method) streptococci distributed among serogroups A, C, G, H, and L. Lysis-from-without by excessive numbers of phage, and enzymatic lysis, were ruled out. Nevertheless, not all these phages were propagable in the strains initially lysed, for

reasons yet unclear. However, 4 phages (2 Group A, 1 Group E, 1 Group G) were propagated in a streptomycin-resistant Group A donor streptococcus. All were subsequently capable of transducing the streptomycin-resistance marker to other strains of Group A streptococci, but only the Group A phages transduced a heterologous recipient (Group C). This is the first known instance of intergroup transduction by phages originating in other streptococcal serogroups. The results raise many questions of specificity of phage receptors and host restriction mechanisms. Active study of similar transductions of other markers is in progress. (Colon, Cole, Leonard)

Discovery and Genetic Importance of "Group H" Streptococcal Bacteriophages

Bacteriophages originating from, or capable of infecting, those streptococci, loosely classified as serologic Group H were previously unknown. Since "Group H" includes the genetically transformable streptococci, the lack of phages precluded essential adjunct genetic studies such as phage-mediated transduction or transfection by isolated phage DNA. Such methods, available in genetic systems in other bacteria, are invaluable in chromosome mapping, clarification of DNA restriction mechanisms, and in determining the nature of competence for genetic transfer. Now, by mitomycin C induction, 5 of 17 "Group H" streptococci have been found to be lysogenic, and several strains can act as propagating hosts which can also be lysogenized. Transfection with phage DNA was shown. In addition, when highly-transformable strains are lysogenized, they lose transformability although retaining the capacity to make or respond to competence factor. The latter was determined by the ability of such cells to complete "early" stages of competence which terminate in an autolytic phenomenon. By use of radiolabeled DNA, failure of transformation was shown due to complete failure of DNA binding or uptake, thus placing the defect at a "late" stage of competence. Studies to define the cellular basis for this defect are in progress. (Parsons, Colon, Leonard, Ranhand, Cole)

Nature and Role of Streptococcal Competence Factor Clarified

New and improved methods augmented prior data on the characterization of the factor (CF) which is produced by, and induces genetic competence in, Group H streptococci. The substance is of low molecular weight, dialyzable, thermoresistant, and contains no lipid, phosphorus, nor carbohydrate. It is 90% protein (Lowry-reacting material) with 15% organic nitrogen, and is highly basic: its isoelectric point is greater than pH 11.0. This latter, plus its trypsin-sensitivity, precipitability by trichloroacetic acid, and the absence of sulfur-containing amino acids, suggest that it may be a polyamine--but not a free one. The highly basic nature suggests possible relations between binding of acidic transforming DNA and alterations between binding of acidic transforming DNA and alterations of the negatively-charged bacterial surface. In fact, continuing biological studies show that CF, though present early intracellularly, must be released and then re-bound to cells of a specific physiological age, in order to induce the steps leading to genetic competence and DNA uptake. The induction requires 40 minutes of uninterrupted protein synthesis after CF binding, and the new proteins produced and the specific sequence of steps involved, are under study. (Leonard)

Competence-Associated Autolysin Not Specific for Group H Streptococci

It was previously shown that genetically competent, but not non-competent, cultures of Group H streptococci produce an autolytic enzyme which splits the walls of both competent and non-competent cells only at the growing cell equator. This restricted and limited activity raises questions of substrate specificity as well as of enzyme nature. A first step in answering these was to test the enzyme against streptococci of 16 different serological groups. Streptococci from Groups A, E, H, and K (one strain) were lysed; those from Groups B, C, D, F, G, K (one strain), L, M, N, P, Q, R, and S, were not. Strains of other Gram-positive bacteria (Bacillus, Micrococcus, Staphylococcus, Lactobacillus) were not lysed. In all positives, lysis was restricted to the cell wall equator. The results point out previously unrecognized differences or similarities in cell wall composition among streptococci, or even between strains presumably of a common serogroup (K). The unique ability of the autolysin to attack only newly-synthesized cell wall is being explored by purification of the enzyme. (Ranhand, Cole)

Reasons for Non-degradation of Group A Streptococcal Cell Walls in Human Leukocytes

Neither monocytes (last year's report) nor polymorphonuclear leukocytes (this report) from human peripheral blood can degrade native Group A streptococcal cell walls--as determined by electron microscopy. The walls of whole cells, or mucopeptide shells thereof, were unaffected by intraphagosomal residence in vivo, or by leukocyte granule extracts or lysozyme in vitro. The wall mucopeptide is known to possess a large number of free amino groups, which are known to inhibit lysozyme activity; but its hydroxyl (O-) groups are also free and hinder lysozyme only when substituted (as by acyl-groups). We treated walls by methods (formamide, acid) that removed polysaccharide. Formamide also substituted the free NH_2 and O groups; after acid, this was accomplished with acetic anhydride. Subsequent saponification by alkali de-acylated the O-groups only. In this state the walls (mucopeptide) were completely degraded by lysozyme, leukocyte granule extracts, and within phagocytic vacuoles. The results indicate that, although the leukocyte possesses lysozyme-like enzymes, they are ineffective because the native streptococcal wall possesses a mucopeptide which is enzyme-insensitive because of its chemical conformation. These findings supply a probable explanation for the previously-demonstrated persistence of, and lesion-production by, Group A streptococcal walls in rabbits, and suggest that the pathogenesis of post-streptococcal disease in man could be related to a similar phenomenon. (Glick, Ranhand, Cole)

Medical Mycology Section

Sexual State of Histoplasma capsulatum Discovered

Long searched for, and once erroneously reported, the sexual state of this fungus has at last been found. Mating was first demonstrated in strains isolated in Texas, but further work showed that isolates, regardless of their origin, occur in two mating types (+ and -). When compatible strains are paired on Alphacel-yeast extract-agar, sexual fruiting bodies containing ascospores are produced. This discovery of heterothallism now provides means for fundamental genetic studies of this human pathogen. (Kwon-Chung)

Interferon Stimulator Enhances Susceptibility of Mice to Some Experimental Fungal Infections

Mice injected with Polyinosinic: Polycytidylic acid (In:Cn) are more susceptible to candidiasis and coccidioidomycosis than are untreated controls. Increased susceptibility is manifested by an accelerated fungal growth in tissues and a higher rate of mortality. Mice infected with Cryptococcus neoformans are not similarly affected by In:Cn. Studies in this laboratory and by other investigators also show an increased susceptibility to fungal infection by animals receiving other cytotoxic and immunosuppressive agents. Patients receiving antitumor therapy, and those immunologically suppressed, have a high incidence of secondary fungal infections. Since In:Cn is an antitumor agent in animals, and may be used in man, it also may be expected to enhance infectious sequelae. (Hasenclever)

pH May be an Important Factor in the Survival of Histoplasma capsulatum

Histoplasma capsulatum does not survive under laboratory conditions for more than six weeks if the pH of the soil medium is less than five. When cultural conditions favored the development of a neutral or slightly alkaline environment, the fungus survived well regardless of the microbiological flora present. Generally a predominant soil flora of bacteria, rather than other fungi, induced alkaline conditions and better survival or growth of Histoplasma. However, the additions of bat guano or additives that stimulate bacterial growth and subsequent alkalinity in the laboratory have not resulted in measurable repopulation by H. capsulatum of circumscribed areas in the field. The ecology of this fungus, and means for its natural control, are problems requiring continued study. (Hasenclever, Kwon-Chung)

Yeast Flora of Birds Much Like that of Humans

Most of the species of yeasts that constitute a portion of the oral and gastrointestinal microbial flora of columbiform birds are the same as in human beings. The common pigeon, Columba livia, was studied most extensively, and Candida albicans was the yeast isolated most frequently. The ratio of the antigenic groups within the avian isolates is about the same as for humans. Other yeasts isolated were C. guilliermondis, C. parapsilosis, and Torulopsis glabrata. Any of these yeast-like fungi may cause serious, though usually secondary, disease in human beings. There is no evidence to suggest any exchange of these yeasts between man and birds but the constant presence of feral pigeons in the urban environment must be considered. (Hasenclever)

Mycoplasma Section

New Acholeplasmas

Our earlier studies on sterol-nonrequiring mycoplasmas provided the basis for a new taxonomic grouping for these organisms (Acholeplasma) and a new species in this taxa was described. Studies the past year confirmed our initial impression that the acholeplasmas represent a large group of distinct organisms that differ fundamentally from species of Mycoplasma. On the basis of several biochemical and serological properties, we characterized approximately 15 new acholeplasmas isolated from a variety of hosts. These organisms

appear to fall into [illegible] distinct groups, three of which have some serological relationship to previously described Acholeplasma species but differ in some minor biological markers. The remaining two groups seem to be more unique, since they are unrelated serologically to each other and to other acholeplasmas, and possess some distinct biochemical markers. The widespread host distribution of these acholeplasmas and their frequent association with animal diseases (conjunctivitis, pneumonia) suggest that they can provide insight into an understanding of the pathogenicity of sterol-nonrequiring mycoplasmas. (Tully)

Mycoplasmas and Mixed Infections

Studies on a paralytic syndrome associated with an experimental Plasmodium berghei infection in rats have revealed indirect evidence that Mycoplasma neurolyticum is involved in the neurological aspects of the syndrome. The mixed infection in the rat appears to involve a delicate interaction of malaria parasites, mycoplasmas, and mycoplasma neurotoxin. The lethal outcome of this interaction is apparently dependent upon a specific sequence of events originating with a developing malarial infection, stress of the host, enhancement in the numbers of neurotoxic mycoplasmas and, finally, accumulation of sufficient amounts of neurotoxin to produce paralysis. The failure to recover M. neurolyticum from rats with either the experimental mixed infection or an artificially-induced infection with neurotoxic mycoplasmas and a non-paralytic malarial strain, raises important questions about the interaction of the host response and mycoplasmas in the development of latent and mixed infections. An understanding of how host-adapted mycoplasmas can be induced to grow in culture will be of considerable importance in extablishing their role in human diseases of unknown etiology. (Tully; Mercado, LPD)

Biological and Serological Characterization of New Animal Mycoplasmas

The characterization of various animal mycoplasmas reported last year has confirmed the serological interrelationship of a group of mycoplasmas isolated from outbreaks of keratoconjunctivitis in two separate sheep and goat herds. Further analysis of the strains by acrylamide gel electrophoresis, sterol requirements, biochemical properties, and their serological characteristics indicated that these mycoplasmas were dinstinct from other established species. A description of the properties of these strains and a new species designation of M. conjunctivae has been proposed. An additional group of seven mycoplasmas representing isolates from man and several other animal hosts appears to be related to some pathogenic goat mycoplasmas. Confirmation of the different host distribution of these organisms and their possible role in human disease are under study. (Tully; Barile, LBP-DBS)

Immunology Section

"Allergic" Renal Tubule Disease in Guinea Pigs

The role of bacteria as the source of inducing antigens or of adjuvants in the pathogenesis of post-infectious kidney diseases in man still awaits clarification. "Allergic" renal tubule disease of guinea pigs was induced by rabbit renal tubule antigens incorporated into various adjuvants. This disease was strain and age dependent, with resistance in Strain 2 and older guinea pigs. Young Strain 13 animals were regularly susceptible. Transfers of

sensitized lymph node cells to histocompatible normal guinea pigs led to glucosuria in some recipients of ten-day, but not five or seventeen day lymph node harvests. (Stone)

Pathogenesis of Chronic Autoimmune Encephalomyelitis

An age, sex, and strain dependent chronic autoimmune encephalomyelitis in guinea pigs is providing a better analogy to human demyelinating diseases than the acute disease usually seen in this species. Strain 13 guinea pigs, which regularly develop the chronic disease when injected with isologous spinal cord in complete Freund adjuvants at an early age (100-250 g), show the first signs of paralysis usually after two months latent period. During this time, the guinea pigs are evidently protected against the acute disease since attempts to actively or passively (with lymph node cells from sensitized adult donors) induce acute autoimmune encephalomyelitis do not succeed. (Stone)

Studies on Cellular Manifestations of Hypersensitivity

Immune deviation phenomena indicate the importance of the first adjuvant stimulation accompanying the primary infection of a given antigen. The mode of immune repsonse favored by the initial adjuvanticity may dominate all subsequent attempts to induce other manifestations of the immune response to that antigen. Thus, the original injection of alum-precipitated bovine gamma globulin leads to an inhibitory state resisting subsequent attempts to induce delayed hypersensitivity to that antigen which lasts for up to nine months. This resistance extends to attempts to sensitize using as much as 5mg of killed Mycobacterium tuberculosis in the adjuvant. Alum-induced deviation prevents induction of Jones-Mote or cutaneous basophil hypersensitivity as well as the classic manifestations of delayed hypersensitivity. (Stone)

Serial No. NIAID 61
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Electron Transport System in an Autotrophic Hydrogenomonad and the Effect of CO_2 on Growth

Previous Serial Number: Same

Principal Investigator: Dr. Roy Repaske

Other Investigator: Guest Worker - Mrs. Anne C. Repaske

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	22/12	-	6/12	28/12
Professional	12/12	-	6/12	18/12
Other	10/12	-	-	10/12

Project Description:

Objectives:

1. Compare the effect of CO_2/bicarbonate on growth of selected heterotrophic bacteria; i.e., E. coli (aerobic, simple inorganic medium), a Group H Streptococcus (anaerobic, complex organic medium); in relation to the effect on growth of H. eutropha (aerobic, simple inorganic medium).

2. Determine effect of CO_2/bicarbonate on synthesis of transforming factor and development of competence in Streptococcus (Challis). (Joint research with C. Leonard).

3. Examine trace mineral requirements for growth of H. eutropha.

4. Characterize and purify enzymes of the electron transport system of H. eutropha.

Methods Employed:

The method employed to discriminate between a CO_2 or a bicarbonate requirement for cell growth was based upon the application of the Henderson-Hasselbalch equation which relates the partial pressure of CO_2 to bicarbonate ion concentration as a function of pH. The percentage composition of CO_2 and other gases in the atmosphere over the fermentor culture were extablished by flow meters. Gas flow in excess of needs assured a constant gas composition.

The method employed to detect micronutrient requirements of Hydrogenomonas eutropha depended upon depletion of trace amounts of micronutrients contained as contaminants in the major nutrients. Depletion could be effected only by growing high density culture populations in a defined medium and observing a change in the growth rate. Suspected micronutrients were added until the original growth rate was reestablished.

Major Findings:

1. Last year we reported that growth of Hydrogenomonas eutropha was dependent upon the bicarbonate ion concentration, not upon CO_2 concentration. This was shown after determining the optimum CO_2 concentration at a given pH and showing the adverse effect of increasing the pH of the medium while the CO_2 was kept constant (bicarbonate ion concentration increased). At the higher pH, an appropriate decrease in the concentration of CO_2 reestablished the previous bicarbonate ion concentration and simultaneously the growth rate returned to its original maximum rate. A series of these experiments clearly showed that the absolute CO_2 concentration was not important per se except to indirectly provide bicarbonate whose concentration was critical for growth.

Experiments by others in the early 1930's showed that, without exception, all of 100 different species of microorganisms tested required CO_2 to initiate growth. We confirmed the CO_2 (bicarbonate) requirement for aerobic growth of E. coli in a defined simple medium, and for anaerobic growth of Streptococcus (Challis) in a complex medium. Neither organism initiated growth unless CO_2 was provided in the atmosphere. After two hours incubation in a CO_2 free atmosphere, during which there was no growth, both organisms grew immediately upon addition of CO_2. Cell growth could be regulated at will be exhausting or adding CO_2 to an otherwise complete medium. When the concentration of cells became sufficiently high, removal of CO_2 no longer stopped growth. Presumably endogenously produced CO_2 satisfied the CO_2 requirement.

The rate of growth was dependent upon the CO_2 concentration which was characteristic for each organism. Growth rates for E. coli, however, were the same at pH 6.4 and at pH 7.0. The absence of a pH effect indicated E. coli responded to CO_2, but not to bicarbonate concentration. Streptococcus, on the other hand, required less CO_2 as the pH of medium was raised. It was not sufficiently less to attribute growth exclusively to a bicarbonate ion effect. The data fit the postulate that Streptococcus (Challis) requires CO_2 as well as bicarbonate for growth.

Thus, three diverse organisms, an autotrophic aerobe, a facultative heterotrophic anaerobe and a heterotrophic anaerobe, have been shown to depend upon either CO_2 or bicarbonate or both for growth. All three can be grown without a lag period, even from old stationary inocula, if the proper CO_2/bicarbonate concentration is provided.

2. Another series of experiments conducted cooperatively with C. G. Leonard were designed to determine if a CO_2 (bicarbonate) requirement exists for the production of competence factor and development of competence in Streptococcus (Challis). Results thus far show CO_2/bicarbonate is needed for synthesis of transforming factor and for cell competence.

3. Micronutrient requirements for H. eutropha have been shown for iron and nickel, and there is an uncomfirmed report of a zinc requirement. These and other common micronutrients are routinely added to the medium. Even with these additions, after cultures reached a cell concentration of about 2 X 10^{10} cells per ml, a marked decrease in growth rate occurred. The change in rate was prevented by a supplement of $MgSO_4$ but not $MgCl_2$, indicating a trace contaminant in $MgSO_4$ was responsible for the increase in rate. Spectroscopic analysis showed $MgSO_4$ contained chromium in relatively high concentration. Chromium salts replaced $MgSO_4$ as a growth stimulant. Two other cations, copper and cobalt, have also been found to be stimulatory for growth of H. eutropha. It is difficult to demonstrate a clear cobalt effect because it is needed in very low concentrations (about 1 X 10^{-8} M); and nickel, which is also required, contains about 0.1% cobalt as a contaminant.

4. Purification of electron transport system enzymes is continuing. The enzyme which transports electrons from reduced pyridine nucleotide to the next intermediate in the system seems to be NADH sytochrome c reductase. The enzyme was extensively purified. Most other bacterial cytochrome c reductases transfer electrons only to cytochrome c derived from the same species; the enzyme from H. eutropha can function with mammalian cytochrome c.

Interpretation of the role of NADH cytochrome c reductase as a direct reductant of cytochrome c remains open because another cytochrome found in the organsism, cytochrome b, would have no function. Ordinarily, cytochromes of the "b" type have a lower potential than cytochrome c, therefore, cytochrome b should be reduced before "c". An additional consideration is that cytochrome b is found associated with membrane fragments of the cell, whereas cytochrome c is soluble. It is possible that cytochrome c reductase is a bypass system used when "normal" electron transport through "b" is to be avoided.

Significance to Bio-Medical Research and the Program of the Institute:

Some form of CO_2 seems to be required by all microorganisms to initiate and to continue growth. The effect is particularly apparent in cultures freshly inoculated with old inocula whose metabolic activity is low and endogenous CO_2 is not rapidly produced. A simplified defined medium is most useful for demonstrating a CO_2 (bicarbonate) requirement for growth of a culture, presumably because endogenously produced CO_2 is minimal.

The universal requirement for CO_2 in some form to initiate growth, though known for some time, has not been exploited. The current demonstration of a continued requirement to maintain growth now places CO_2 in a central position as a key regulator of cell growth. This was shown with a representative aerobic organism (E. coil) and an anaerobic organism (Streptococcus (Challis)). The Streptococcus example is especially significant since this organism is one of the homolactic lactic acid organisms which produce only traces of CO_2 during metabolism.

Proposed Course of Project:

A) Culture Studies -

Determine the optimum percentage CO_2 and the optimum bicarbonate concentration required for Streptococcus (Challis). With Streptococcus and with E. coil use C^{14} labeled CO_2/bicarbonate to identify the key CO_2/bicarbonate fixation reaction regulating growth. If CO_2/bicarbonate is not incorporated into some cell product, it would suggest CO_2/bicarbonate is functioning as an activator for some enzyme critical for initiation of cell growth.

Determine the species of carbon required by Streptococcus (Challis) for (I) developing competence (II) synthesis of competence factor.

Conclude H. eutropha growth experiments related to nutritional requirements for maximum growth rates and yields.

B) Identify and purify enzymes of the electron transport system of H. eutropha.

Attempt to determine the role of NADH cytochrome c reductase as a bypass or as a direct step in normal electron transport.

Honors and Awards:

Convener: Oxidoreductases Session, American Society for Microbiology, Annual Meeting, April 26, 1972, Philadelphia, Pennsylvania.

Publications:

Repaske, R., Ambrose, C. A., Repaske, A. C., and DeLacy, M. L.: Bicarbonate Requirement for Elimination of the Lag Period of Hydrogenomonas eutropha. J. Bacteriol. 107: 712-717, 1971.

Serial No. NIAID 62
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Studies on Staphylococcal and Streptococcal Cell Walls

Previous Serial Number: Same

Principal Investigator: Dr. Eskin Huff

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To develop methods for the quantitative determination of polyols (glycerol, ribitol, anhydroribitol, erythritol), sugars (rhamnose, ribose, fucose, xylose, galactose, glucose, mannose), hexosamines (galactosamine, glucosamine, mannosamine), and amino acids (muramic acid, alanine, glycine, aspartic acid, glutamic acid, lysine, diaminopimelic acid) in hydrolysates of streptococcal and staphylococcal cell walls. Our goal is to compare strains that differ in a) sensitivity to lysis, b) level of autolysin and c) presence of lysogenic phage serology - to see if cell wall structure change accompanies changes in lytic susceptibility or differences in immunogenicity.

Methods Employed:

Isolation of cell walls and growth of cells has been previously described. One to two milligrams of dry cell wall material is sealed in a 6 X 50 mm

testtube with 50 microliters of acid (2N, 4N, or 6N HCl or H_2SO_4) under argon. The tube is placed in a silicone oil bath at 100°C for 2 to 20 hours. Sugars and polyols are separated from charged molecules by passing the hydrolysate through a Dowex 50 H+ column on top of a Amberlite IRA 410 acetate column. Amino acids and hexosamines are eluted from the Dowex 50 H+ with 4N HCl.

The sugar and polyol fraction is a) treated with sodium borohydride to reduce the sugars to polyols, b) treated with acetic acid to convert excess borohydride to borate, c) dried under argon after adding methanol to remove borate. The entire mixture is then acetylated with acetic anhydride and pyridine, dried, and then injected in chloroform onto an 8 foot stainless steel column packed with 3% ECNSS-M (cyanoethyl silicone) on Chromosorb Q in the gas chromatograph.

The amino acid fraction is lyophilized, propylated with a mixture of propanol and HCl, dried under argon, and acylated with a mixture of heptafluorobutyric acid anhydride and pyridine at 110°C for 15 minutes. It is then dried with a stream of argon, dissolved in chloroform, extracted with water 3 times, and injected in chloroform onto a 12 foot glass column packed with 1% OV-17 (phenylmethylsilicone) on Chromosorb Q in the gas chromatograph. Cell wall amino acids and muramic acid are separated by this means. The amino sugars are complicated, with multiple peaks, but they do not interfere with muramic acid or the amino acids.

Major Findings:

By using specially selected and washed ion exchange resins, redistilled methanol (stored under argon to prevent oxidation), and by drying down methanol with a stream of argon when removing methyl borate, it has been possible to prepare alditol acetates of polyols and sugars on a micro scale for gas-liquid chromatographic analysis. Alditol acetates are very stable in contrast to the previously used trimethylsilyl derivatives. Separation and quantitation of glycerol, erythritol, anhydrorititol, rhamnose, ribitol, xylose, deoxyglucose, mannose, galactose and glucose and many other sugars and polyols has been achieved. As little as 10 nanograms of sugar can be detected (10^{-5} milligrams). Since the yield from 1 milligram of sample is taken up in 20 microliters of chloroform and we usually inject 1 microliter into the gas chromatograph, the overall sensitivity is in the range of 0.02% of the starting dry weight.

Instability of the trimethylsilyl amino acids, which we have used previously, led us to seek a more reliable method. We have adapted a recently described method in which the amino acids are converted to their propyl esters and then treated with heptafluorobutyric acid anhydride. This procedure has the advantage of allowing separation of the usual amino acids found in the cell wall; in addition, muramic acid forms only one major derivative which separates from other amino acids. These derivatives are also quite stable.

The cell walls from 13 Group H Streptococci and 3 Staphylococcus aureus strains have been hydrolyzed and examined for neutral carbohydrate content. With the exception of three streptococcal strains which vary from three strains only by being lysogenic, each strain examined had a unique carbohydrate analysis.

One strain (FW-35), which was immunologically unlike either of the known two subgroups of Group H streptococcus, had 1% of an unknown carbohydrate. Three strain pairs in which the members of the pair differed by being lysogenic or non-lysogenic (phage sensitive), showed no differences in carbohydrate content. In one of these strains it has been recently shown (Project No. NIAID 65A-1, Parsons and Ranhand) that no DNA binding nor uptake occurs in the lysogenic member of the pair, whereas the non-lysogenic strain is genetically fully competent. This difference can not be accounted for by differences in the cell wall neutral carbohydrate composition.

Other materials analyzed for neutral carbohydrate were a) purified competence factor (Project No. NIAID 65-C, Leonard) from Streptococcus Group H strain Challis (no carbohydrate detected) b) immunologically active lipid (Project No. NIAID 64-D, Prescott) from a Group D Streptococcus (2.7% glycerol; 19.0% glucose); c) mesosomes (6.4% glycerol; 1.2% glucose) and membranes (1.4% glycerol; 0.6% ribitol; 1.0% glucose) from Staphylococcus aureus (Project No. NIAID 63B; Theodore).

Significance to Bio-Medical Research and the Program of the Institute:

New microtechniques using ion exchange and gas-liquid chromatography have been further developed and applied to the analysis of acidic, basic and neutral components of bacterial cell walls and other components. These methods are important in analysis of bacterial subfractions, knowledge of which is essential to the coordinated program of the Laboratory of Microbiology. The rapidity, sensitivity and quantitative nature of these methods are vital to our continued chemical study of the cell wall fragments released from streptococcal and staphylococcal cell walls by lytic enzymes and autolysins. This study has been restricted because of time required to develop the essential analytic methods described.

Proposed Course of Project:

Streptococcal and staphylococcal cell walls will be examined further for neutral carbohydrates and amino acids. In addition, a newly reported method for the separation of hexosamines as their cyclic butane boronates (Frank Eisenberg personal communication) will be used to analyze these cell walls for glucosamine, galactosamine, and mannosamine.

In an effort to determine the nature of the autolysin causing the rapid autolysis in Staphylococcus aureus 968, the cell walls and previously isolated subfractions of autolyzed 968, as well as cell walls of 968 mutants will be analyzed for carbohydrate, amino sugar and amino acid content.

Honors and Awards:

None.

Publications:

None.

Serial No. NIAID 63A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Occurrence, Properties and Significance of Protein Iron-Chelators in the Biochemistry of the Acquisition of Iron, and Possibly Other Metals, by Microbial and Animal Cells

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	12/12	-	-	12/12
Professional	4/12	-	-	4/12
Other	8/12	-	-	8/12

Project Description:

Objectives:

To determine the occurrence, properties, and significance of protein iron chelators and to investigate the mechanisms by which microbial and animal cells acquire from plasma siderophilin, ekkrinosiderophilin, and conalbumin the iron and possibly other metals for normal growth and function and the effect of physiological conditions upon this exchange.

Methods Employed:

Hematological procedures, radioactive tracer techniques, manometric techniques, strip and disc electrophoresis, chromatography, optical rotatory dispersion, infrared spectroscopy, sucrose gradient sedimentation, and immunologic analyses.

Major Findings:

Having previously established that the iron-binding protein of circulating blood, plasma siderophilin, effects bacteriostasis of Staphylococcus epidermidis and Staphylococcus aureus through nutritional iron deprivation, we have now found that the iron-binding protein of human milk and other body secretions, ekkrinosiderophilin (lactoferrin), is similarly effective in inhibiting the growth of these pathogens. Studies of growth rates by turbidity measurements and viable-colony counts have shown that the percentage iron-saturation of ekkrinosiderophilin added to vitamin-fortified trypticase broth profoundly affects the cell population increase of S. aureus. For example, at observed maximum growth rates, the generation times of cultures in media containing ekkrinosiderophilin at 90, 60, and 15% iron-saturation were 39, 56, 311 minutes respectively. By turbidity measurements of S. aureus cultures at λ600nm, those with ekkrinosiderophilin at 90, 60, and 15% iron-saturation showed a visible growth delay of 5, 30, and 42 hours respectively, compared to those grown in the absence of ekkrinosiderophilin. The ekkrinosiderophilin concentration employed was equivalent to that of plasma siderophilin in blood (2.5 mg/ml), and roughly one-half to one-third its concentration in mother's milk during the 5-10 days post parturition. The ekkrinosiderophilin in such milk is almost entirely iron-free.

In pursuit of a probable biological or physiological function for the presence in human milk of relatively high concentrations of ekkrinosiderophilin during the period immediately following parturition, we obtained 23 serum samples from 14 male and female newborns 1 to 15 days post parturition. The samples were generously supplied by Prof. W. Auerswald, head of the Dept. of Physiology, Univeristy of Vienna Medical School. Immunological analyses by micro-Ouchterlony plate methods, employing antisera raised in rabbits versus whole human milk and its isolated ekkrinosiderophilin, failed to reveal any proteins peculiar to milk in the infants' sera. The antisera used had been absorbed by concentrated, pooled human serum. However, 3 different cord blood samples gave one similar precipitation line against the whole milk, serum-absorbed antiserum. This precipitation line did not appear when the serum of cord blood was set against the anti-ekkrinosiderophilin serum or against whole milk antiserum absorbed with whole milk as well as with pooled human serum. Thus, the results clearly indicate that some peculiarly milk protein passes the placenta into the foetal circulation; that the protein is not the iron-binding ekkrinosiderophilin; and that it does not survive in the circulating blood of the newborn.

A sample of milk ekkrinosiderophilin, isolated by us from human milk through a previously reported procedure of DEAE cellulose column fractionation at pH 7.4 with increasing ionic strength of eluant followed by G-150 Sephadex column chromatography, was found to be complexed with at least one other protein plus a trace of lysozyme activity. The latter point is of interest since it is now generally the experience that, wherever lysozyme is found, ekkrinosiderophilin likewise is present. Since the antimicrobial activities of both factors, present in the array of bodily secretions and in the neutrophilic leucocytes as well, could be complementary, it is most desireable to have available monospecific antiserum active solely against ekkrinosiderophilin and not possibly active against some other protein with which it is complexed. Dr. B. Reiter of the National Institute for Research in Dairying of Reading, England, tested not

only our ekkrinosiderophilin preparation but one isolated by him in crystalline form with another procedure and found similar centaminants in both. From these results, it appears improbable that an antiserum monospecific for ekkrinosiderophilin has yet been produced. We therefore concentrated on the means of freeing our isolated ekkrinosiderophilin from the protein(s) with which it is complexed. We employed, without success, DEAE fractionation with a combined borate-phosphate buffer procedure found by others to effect separation of hemopexin from plasma siderophilin. Hydroxylapatite adsorption at pH 6.8 was employed without effecting separation of the protein components. Since electrophoresis of our starting material on cellulose acetate at pH 4.0 gave separation of two proteins, one iron-binding and the other not, we employed preparative zonal electrophoresis on polyvinyl chloride at pH 4.0 to obtain larger quantities of the iron-binding moiety. The attempt was only partially successful since endosmosis permitted too little separation of ekkrinosiderophilin free of contaminants. We then prepared a Sephadex sulfonic ester cation exchange column at pH 4.0, ionic strength $\Gamma/2$ of 0.12, and eluted with increasing ionic strengths (NaCl additions) of 0.12-molar acetate buffer at the same pH to an approximate value of $\Gamma/2 = 1.0$. Good protein separation was achieved but the yield was poor. Finally, we used the milder carboxymethyl cellulose cation exchange column at pH 5.95 with a beginning $\Gamma/2$ value of 0.01. The proteins were eluted from the column by a continuous gradient of sodium acetate solution up to a $\Gamma/2$ value of 0.5. Three components were separated. The iron-binding fraction was eluted over the $\Gamma/2$ range of 0.215 to 0.5. Cellulose acetate electrophoresis of the latter fraction at a series of pH values from 4.0 to 10.5 showed only one component. Acrylamide gel electrophoreses at acid and alkaline pH's likewise show only a single band and thus attest to a high degree of purity of the isolated protein. Determination of its isoelectric point (pI) is dramatically dependent upon the composition of the buffers employed. Using zwitter ionic buffers, the pI is approximately pH 10.5; in phosphate buffer, the value is ca. pH 6.0; in citrate it is ca. pH 5.15. No pI could be established in acetate buffer, but is demonstrably well above pH 6.27. It is known that ekkrinosiderophilin interacts strongly with more acid macromolecules such as albumin or casein but not with lysozyme which shares with this protein a very high isoelectric point. What significance the cationic property of these two antimicrobial substances has for their association and possibe participation in immune phenomena remains to be explored.

Significance to Bio-Medical Research and the Program of the Institute:

The presence of protein-iron chelators in biological fluids which have contact with the exterior environment as well as in those providing the intimate milieu of host organs and their constituent cells is a regulatory factor in the iron, and possibly other metals, nutrition and metabolism of both host and parasite, and consequently in their mutual relationships. In addition to determining where and in what concentrations such chelators are found in biological fluids, it is desireable to establish the conditions governing the availability of the metal and the mechanism of its transfer to host tissues and to the offending parasite. The results of such investigations involving nutrilites, such as iron, so essential to the life and metablism of both host and parasite, should increase our understanding of infection and its control.

Proposed Course of Project:

We propose to investigate further the purity of our isolated ekkrinosiderophilin (lactoferrin) of human milk by electrophoretic and immunological procedures; to refine, if necessary; and to relate the physico-chemical properties of the definitive product to its microbiological and/or physiological significance to man.

Honors and Awards:

None.

Publications:

Kirkpatrick, C. H., Green, I., Rich, R. R. and Schade, A. L.: Inhibition of growth of Candida albicans by iron-unsaturated lactoferrin: relation to host-defence mechanisms in chronic mucocutaneous candidiasis. J. Infect. Dis. 124: 539-544, 1971.

Serial No. NIAID 63B
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the nutrition, physiology and ultrastructure of Staphylococcus aureus.

Previous Serial Number: Same

Principal Investigator: Dr. Theodore S. Theodore

Other Investigators: Dr. Roger M. Cole and Mr. Terry J. Popkin

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

To isolate and characterize by cytological and biochemical methods the membrane system of Staphylococcus aureus; more specifically, to examine the distribution of certain enzymes and compounds in the membrane system in order to allow for the biochemical, physiological and histological differentiation of mesosomes and plasma membrane.

Methods Employed:

Routine bacterial and cultural procedures; spectrophotometry, spectrophotofluorometry, paper and thin layer chromatography, polyacrylamide gel electrophoresis and routine metabolic, enzymatic and radioisotope procedures; differential and density gradient centrifugation techniques and electron microscopy.

Major Findings:

Previous studies on the separation and isolation of membranes and mesosomes from S. aureus ATCC 6538P; and also, electron microscopy studies on the release and purification of these two membrane fractions have been completed and published. This year, we have devoted our time to (A) large scale fractionation of mesosomes and isolation of cleaner membrane fractions, (B) protein and lipid characterization of membranes and mesosomes, and (C) cytochemical localization of respiratory activities in the whole cell.

(A) We initiated large scale preparation of mesosomes to obtain sufficient material for biochemical and enzymatic analyses. This need was obvious because mesosomes constitute only 1-2% of the dry weight of the staphylococcal cell. By using high cell densities in the protoplasting buffer and concentrated muralytic enzyme, we were able to attain a 10-fold increase in mesosomes (i.e., from 5 mg to 50 mg of mesosomal protein). A major improvement in this large scale fractionation technique was the omission of Mg^{++} during protoplast lysis. Membranes prepared in this manner were free of residual mesosomes and ribosomes; and produced only one homogeneous band after sucrose density gradient centrifugation. (In the absence of Mg^{++}, mesosomes disaggregate, and ribosomes can be degraded with ribonuclease.)

(B) Purified membranes and mesosomes were solubilized in 1% sodium dodecyl sulfate (SDS) and electrophoresed on 0.1% SDS-containing gels. Gel patterns of both membrane fractions showed approximately 26 well-resolved bands of protein. Removal of lipids by chloroform-methanol extraction prior to SDS treatment increased the resolution of the protein bands without altering the gel patterns. Unlike the phenol-acetic acid-urea gel patterns (see last year's report), no qualitative differences between membranes and mesosomes were seen. Quantitatively, membranes contained a higher concentration of high molecular weight proteins while the mesosomes contained a higher concentration of low molecular weight proteins. Attempts to characterize these protein differences (structural differences) by selective staining techniques were unsuccessful. No lipoproteins, ribonucleoproteins, iron-containing proteins or glycoproteins were detected in either SDS or phenol-acetic acid-urea extracted proteins. However, when membranes and mesosomes were extracted with lithium diiodosalicylate (LIS, a solubilizing agent shown to selectively extract glycoproteins from red cell membranes) and electrophoresed on SDS gels, one high molecular weight band appeared which stained positively with Schiff-periodate reagent. This band was most pronounced in the mesosomal extracts. Enzymatic analyses directly on polyacrylamide gels was not done since all the solubilizing agents used to disaggregate membranes and mesosomes were also strong protein denaturing agents.

Recently, we discovered what appears to be a new lipid component which is found almost exclusively in the mesosomes. Membranes and mesosomes were extracted with an acidified butanol-pyridine mixture, pH 4.2, and analyzed by thin layer chromatography. In a chloroform-methanol-water solvent system, this lipid had an apparent rf value of 0.22-0.25 and did not co-chromatograph with any of the known lipid standards shown to be present in S. aureus. The lipid could not be extracted with chloroform-methanol (2/1) and it did not

move in a heptane-ethyl acetate solvent system, indicating that it was not a C_{55} isoprenoid alcohol. This lipid gave a positive test for phospholipid and glycolipid; and was negative for amino acids by the ninhydrin spray method. The carbohydrate moiety of this lipid component is glucose since this was the only sugar detectable by gas-liquid chromatography (Dr. E. Huff). At present, the structure and functional activity, if any, of this lipid is unknown.

(C) As reported last year, attempts to show cytochemical localization of respiratory activities in S. aureus have been unsuccessful. Tellurite localization of oxidative activities and localization fo succinic dehydrogenase by either tetranitro blue tetrazolium or distyryl ditetrazolium salts have so far been non-specific.

Significance to Bio-Medical Research and the Program of the Institute:

The membrane system of Gram positive bacteria consists of a plasma membrane and mesosomes. The mesosomes, which are formed by an invagination of the plasma membrane, have been described morphologically; however, their functional role in the cell and their relationship to the plasma membrane is still not known. Our studies are directed toward a basic understanding of the structural and functional interrelationships between membranes and mesosomes of S. aureus. The approach has been to isolate these organelles in a pure and intact state and characterize them by chemical and enzymatic analysis. We have shown both quantitative and qualitative protein differences on polyacrylamide gels between membranes and mesosomes; and just recently, have detected what appears to be a new lipid component found almost exclusively in the mesosomes. This latter discovery is of great interest since in the past we have succeeded in showing what is not present in mesosomes. Presently, we are analyzing native membranes and mesosomes for structural and enzymatic protein differences, and for the new lipid component. It is anticipated that such studies will enable us to ascertain what specific functions reside in these organelles and how they are related to cell growth.

Proposed Course of Project:

The following lines of investigation will be pursued:

1. Isolation and determination of the structure and functional activity of the newly found lipid component in mesosomes.

2. Detailed chemical characterization and enzymatic analysis of protein differences found on polyacrylamide gels. Also, analyses pertaining to cytochrome and lipid biosynthesis, and elements involved in the electron transport chain.

3. Continued attempts at cytochemical localization of respiratory activities.

Honors and Awards:

Presented seminar on "Isolation and Characterization of Mesosomal Vesicles from Staphylococcus aureus," Dept. of Biological Sciences, Marquette University, Milwaukee, Wisconsin, Nov. 1971. (Theodore)

Publications:

Theodore, T. S., Popkin, T. J., and Cole, R. M.: The separation and isolation of plasma membranes and mesosomal vesicles from Staphylococcus aureus. Prep. Biochem. 1: 233-247, 1971.

Popkin, T. J., Theodore, T. S., and Cole,R. M.: Electron microscopy during release and purification of mesosomal vesicles and protoplast membranes from Staphylococcus aureus. J. Bacteriol. 107: 907-917, 1971.

Serial No. NIAID 63C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Investigations of Gram-Positive Cocci

Previous Serial Number: Same

Principal Investigator: Dr. Arthur L. Schade

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	16/12	-	-	16/12
Professional	8/12	-	-	8/12
Other	8/12	-	-	8/12

Project Description:

Objectives:

To investigate the protein composition of Group A streptococcal plasma membranes and mesosomes for identity or non-identity of their structure and function.

Methods Employed:

Standard methods of bacteriology, metabolism, and biochemistry. Special methods as required including a variety of immunological procedures; radioactivity techniques, spectrophotometry, electrophoresis, chromatography, and ultracentrifugal gradient separations.

Major Findings:

We have already reported, following preliminary investigation, that phenol-acetic extracts of plasma membranes and mesosomes of Streptococcus Group A, type 4 differ from one another in that acid polyacrylamide gel electrophoreses

of membrane extracts give evidence of 4 more protein species (24) than are found in mesosome extracts (20). These structures were purified by sucrose gradient separation following protoplasting of the streptococcal cells in 30% raffinose solution with streptococcal Group C muralysin and osmotically shocking the protoplasts to allow exhaustive washing of the plasma membranes formed.

In the present investigation, not satisfied that the protoplasts formed in 30% raffinose solution are completely and effectively burst by subsequent osmotic shocking, we treated the streptococcal cells with muralysin, DNase and RNase in 0.05 M phosphate buffer, pH 6.22, for 2 hours at 37°C at which time the turbidity of the suspension had stabilized at ca. 7% of the starting value. Inspection of the muralysin-digested product by electron microscopy showed no intact walls or protoplasts. The digest was then centrifuged at 3,000 rpm, the precipitate discarded, and the supernatant centrifuged at 10,000 rpm to yield a plasma membrane-containing precipitate and a mesosome-containing supernatant. The membrane precipitate was washed five times with 0.05 M tris-HCl buffer, pH 7.4, until no soluble protein appeared in the supernatant and then sucrose-gradiented with ultracentrifugation in a 50-75% (w/v) sugar gradient. The banded membrane fraction was recovered following a buffer wash. The supernatant mesosomal fraction was buffer-washed twice and sucrose-gradiented on 50-70% (w/v) sugar solutions. A single turbid band resulted at ca. the 55% sucrose level, was recovered and buffer-washed to give a product which by electron microscopy with PTA "staining" showed a uniform population of small sized, often irregularly shaped, membranous structures. On a dry weight basis the protein content, by the Lowry method with bovine serum albumin as standard, was 83% for the plasma membranes and 59% for the mesosomes. Gravimetric lipid analysis of both preparations showed 5-6% and 31% for the membranes and mesosomes, respectively.

The isolated membrane and mesosome fractions were subjected to polyacrylamide gel electrophoreses under a variety of conditions. For example, phenol acetic extracts of these fractions when electrophoresed and stained with amidoschwarz gave 25 bands for the membranes and 21 for the mesosomes. Following ammoniated-acetone delipidation of these preparations and electrophoresis of their phenol-acetic extracts, 27 stained bands were observed from the membrane material and 23 from the mesosomes. Electrophoreses of extracts from the delipidated membranes and mesosomes, made with a mixture of Triton X, mercaptoethanol, and 6 molar urea, resulted in a band pattern of 34 lines from the membrane material and 27 lines from the mesosomes. All of these gel electrophoreses were performed under acid conditions. When non-delipidated membrane and mesosome preparations were solubilized with 0.1% sodium dodecyl sulfate plus mercaptoethanol and acrylamide gel-electrophoresed at pH 7.4, a banded pattern of 20 lines was found for the plasma membranes and 17 lines for the mesosomes. In addition to the qualitative differences in the protein composition of plasma membranes and mesosomes of this streptococcal Group A strain, there were minor quantitative differences among the proteins making up the "familial" pattern of the strain. The consistently greater number of protein species found in the variously analyzed plasma membranes is evidence for their distinctiveness as structures compared to the mesosomes.

Significance to Bio-Medical Research and the Program of the Institute:

Since the major constituents of both the plasma membranes and mesosomes are proteins with both catalytic and structural properties, it is essential to our understanding of the functioning of these organelles that they be identified and characterized as to these properties. Of unique and prime importance for the life and growth of any cell is its possession of a membrane upon whose bounding character its individuality depends and across which its necessary contact with the environment is maintained and regulated. To determine the characteristics of the proteins peculiar to membrane assemblage and dynamic persistence in a commonly solubilizing aqueous milieu is to elucidate a primary basis for the dependence of function upon structure in biology. Specifically, the demonstrated protein differences between membranes and mesosomes are of relevance in the current controversy concerning possible differences in their functions.

Proposed Course of Project:

Employing the experience gained in the isolation and analyses of the proteins of the plasma membranes and mesosomes of Group A streptococcal cells, added to our established competence in protein purification and characterization, we proposed to investigate the problem of isolation and purification of the type-specific M protein antigen from a difficult to type strain of Streptococcus pyogenes such as type 4, whose frequency of identification as the etiologic agent in cases of human streptococcal disease is relatively and significantly high. The relevance of the frequency of implication of type 4 in such disease statistics is that the successful purification of its M protein and the inclusion of this antigen in a polyvalent vaccine for protection of humans against streptococcal disease would enhance the prospective efficacy of such a vaccine. Efforts to realize an effective polyvalent vaccine are clearly of significance to bio-medical research and to the program of the Institute.

Honors and Awards:

Continuing membership: U. S. Civil Service Board, Microbiology Panel.

Member Scientific Advisory Committee, Annual Colloquium, Protides of the Biological Fluids, Brugge, Belgium.

Publications:

None

Serial No. NIAID 64A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies with synthetic potential antifungal and antitumor agents.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	TOTAL
Total	2/12	-	-	2/12
Professional	1/12	-	-	1/12
Other	1/12	-	-	1/12

Project Description:

Objectives:

Further studies on the synthesis of new series of compounds as potential non-toxic chemotherapeutic agents against fungal infections and as anticancer agents.

Methods Employed:

Described in previous reports.

Major Findings:

Antifungal Activity. Several series of compounds synthesized in previous years were tested as potential antifungal agents against Histoplasma capsulatum and Cryptococcus neoformans. In a series of non-toxic hydrazones of 9-hydrazinoacridine, one of the derivatives (5-chlorosalicylaldehyde) was very effective in inhibiting the growth of H. capsulatum in vitro in a dose

of 0.8 µg/ml in broth: two compounds (salicylaldehyde and piperonal) possessed in vitro antifungal activity against the same organism in a dose of 4 µg/ml. The 3,4-dichlorobenzaldehyde derivative inhibited the growth of H. capsulatum in broth in a concentration of 20 µg/ml. Among the hydrazones of N-aminorhodanine, 4 derivatives-- 3-iodobenzaldehyde, 5-chlorosalicylaldehyde, 2-carboxybenzaldehyde, and 5-bromoindole-3-carboxaldehyde-- inhibited growth of H. capsulatum at 20 µg/ml. The compounds were ineffective as antifungal agents against C. neoformans. The compounds are being tested for potential antitumor activity by the Drug Research and Development, NCI.

Significance to Bio-Medical Research and the Program of the Institute:

Numerous non-toxic compounds synthesized in our laboratory have exhibited high chemotherapeutic activity as potential antifungal and antitumor agents in animals. Because of high tolerance and effectiveness, some of these chemotherapeutically active compounds may be useful in chemotherapy of human infections and as anticancer agents.

Proposed Course of Project:

Project terminated.

Honors and Awards:

Invited to participate in a Symposium on Chemistry of Natural Products. University of Connecticut, Storrs, Conn., Aug. 2-3, 1971.

Invited to present a paper "Potential Antimalarial Agents" (V) "Sulfones" at the 7th International Congress of Chemotherapy, Prague, Czechoslovakia, August 23-28, 1971.

Invited to participate in the Mardi Gras Symposium in Organic Chemistry, Louisiana State University, Baton Rouge, Louisiana, Feb. 14, 1972.

Continuing membership: Toxicology Information Program (TIP) at the National Library of Medicine. On Roster of Authorities in Specialized Subtopics in Toxicology.

Publications:

Prescott, B.: Potential antimalarial agents. III. Derivatives of 4,'4'''-sulfonylbis(3-chloropropionanilide). Internat. J. Clin. Pharmacology, Therapy, and Toxicology 3: 138-142, 1970.

Prescott, B.: Potential antimalarial agents. IV. Derivatives of 4,4'-diaminodiphenylsulfone. Internat. J. Clin. Pharmacology, Therapy, and Toxicology 4: 207-211, 1971.

Prescott, B.: Protective effect of certain sulfonic acids on the toxicity of lucanthone. Internat. J. Clin. Pharmacology, Therapy, and Toxicology 5: 301-303, 1971.

Li, C. P., Tauraso, N. M., Eddy, B. E., Prescott, B., and Martino, E. C.: Studies on inhibition of viral oncogenesis. III. Effect of clam extracts and methotrexate on tumor formation in male and female hamsters induced by virulent and attenuated adenovirus-12. Archives fur die gesamte Virusforschung (in press)

Li, C. P., Tauraso, N. M., Prescott, B., Eddy, B. E., Hoye, R. C., Martino, E. C., Caldes, G., and Gorschboth, C.: Cancer Chemotherapy: Direct injections of aqueous clam extracts into rodent tumors. Cancer Research Journal (in press)

Prescott, B.: Antitumor activity of 2,2-hydrazobis (3-chloro-1,4-naphthoquinone) against Walker 256 (intramuscular) carcinosarcoma. J. Med. Chem. 15: 107, 1972.

Caldes, G., and Prescott, B.: A potential antileukemic substance present in Shallot (Allium ascalonicum). Planta Medica (in press)

Serial No. NIAID 64C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Further studies on the preparation, antigenic properties, and elucidation of the structure of a serologically active glycolipid hapten from Mycoplasma pneumoniae.

Previous Serial Number: Same

Principal Investigator: Dr. Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: Laboratory of Infectious Diseases (NIAID 70C)
Dr. Robert M. Chanock
Dr. Helmut Brunner
Mr. Walter James

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	10/12	-	-	10/12
Professional	4/12	-	-	4/12
Other	6/12	-	-	6/12

Project Description:

Objectives:

Further production and purification of a serologically active glycolipid hapten; and, in addition, isolation of a serologically active lipoprotein fraction and a polysaccharide fraction from M. pneumoniae.

Methods Employed:

The methods for the extraction and purification of the serologically active glycolipid hapten have been described in previous reports. Large quantities of purified glycolipid hapten were prepared and reaggregated with lipid-depleted membrane protein from Acholeplasma laidlawii in β-buffer dissolved in deionized water in the presence of a 20 MM mg^{2+}. The reaggregate

was dissolved in deionized water and stored in the deep-frrze for use as a potential immunizing agent in animals. In addition, large quantities of a serologically active lipoprotein were prepared by filtration on Sephadex G-25 chromatographic columns of a water soluble supernatant of sonicated defatted M. pneumoniae organisms. Even though preliminary tests of a polysaccharide isolated from Mycoplasma pneumoniae gave negative results in serological tests with rabbit hyperimmune serum, it was thought worthwhile to test this material for serological activity with hamster antiserum against M. pneumoniae, and as a potential immunogen against experimental infection with M. pneumoniae in these animals. The purified polysaccharide was prepared by two well known procedures: A) phenol extraction of the organisms, and B) an alkaline extraction method used for the preparation of highly immunogenic polysaccharides from pneumococci.

Major Findings:

It was shown that a relationship existed between the glycolipid hapten of M. pneumoniae and a lipoprotein isolated from this organism by column chromatography on Sephadex G-25. Significant levels of growth inhibiting antibody are stimulated in rabbits by the lipoprotein. The activity of the fraction could be ascribed to the lipid component (15%) of the fraction because after lipid removal by mild hydrolysis under reflux in alcoholic ammonium hydroxide, the remaining protein did not fix complement in, nor absorb growth inhibiting activity from, rabbit antiserum active against the lipoprotein. Furthermore, the lipid derived from the lipoprotein exhibited high activity in both tests. The derived lipid and the chloroform-methanol (7:3) glycolipid were subjected to thin-layer chromatography. Chromatographic similarity of the two lipids was established by mixing both. The mixture of the two fractions yielded a chromatographic pattern which indicated that the derived lipid was similar to the glycolipid fraction.

Significance to Bio-Medical Research and the Program of the Institute:

Since it was previously shown that the glycolipid hapten of M. pneumoniae requires a protein carrier to be immunogenic, we have emphasized the study of "hybrid" reaggregates of this glycolipid with lipid-depleted membrane protein of a non-pathogen (A. laidlawii) as a potential immunogen. It now appears that a lipoprotein, of which the lipid moiety seems to be glycolipid, can be isolated directly from M. pneumoniae. This lipoprotein is immunogenic in rabbits, and the serologic activity is directed against the lipid fraction. It may, therefore, prove to be a useful "protective" immunogen against M. pneumoniae infection.

Proposed Course of Project:

The glycolipid (M. pneumoniae)-protein (A. laidlawii) reaggregates are currently being tested for immunogenicity in hamsters, using different doses and routes of administration. The newly-isolated lipoprotein from

M. pneumoniae, as well as the polysaccharide fraction, will be similarly tested in hamsters for stimulation of protective antibodies and for active protection against infection.

Honors and Awards:

Consultant to Vaccine Development Committee, Infectious Diseases Branch, Collaborative Research Division.

Advisor to NIH Library on purchase of chemical journals.

Publications:

None

Serial No. NIAID 64D
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Isolation, separation, and characterization of antigenic lipid hapten(s) from streptococci.

Previous Serial Number: Same

Principle Investigator: Dr. Benjamin Prescott

Other Investigators: Dr. James R. King
Dr. Roger M. Cole
George Caldes
Terry J. Popkin

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	30/12	-	-	30/12
Professional	18/12	-	-	18/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

Continuation of studies to isolate, purify, characterize, and test the lipid hapten(s) from several serologic groups of streptococci (A, B, C, D, E, G, and H); and to determine the common antigenic determinant responsible for serological cross-reactions among the different groups of streptococci studied.

Methods Employed:

Previous studies with glycolipids isolated from Group A and D streptococci in this laboratory revealed their unusually high serological activity in the complement-fixation test and showed cross-reactivity with various rabbit streptococcal antisera. Studies were carried out with strains of additional streptococcal groups, namely, Group B (9925), Group C (26RP66), Group E (6642),

Group G (57x262), and Group H (Challis 71x34). Large quantities of organisms from each group were grown in 15 liters of trypticase soy broth. At the end of the 18 hour period, the organisms were harvested and washed three times with distilled water. In each instance, the lipids were extracted from the organisms with chloroform-methanol (2:1). The overall yields of lipids were as follows: Group B (7.2%); Group C (5.3%); Group E (7.1%); Group G (9.5%); and Group H (7.5%). An interesting feature of this extraction procedure is the facility with which the lipids of any of the streptococcal groups studied can be removed. Several other organic solvent systems (acetone, ethyl acetate) were tried for isolating the active material and abandoned because of poor yields and inactive fractions. The crude chloroform-methanol (2:1) lipid extracts were first freed of non-lipid contaminants by the method of Folch on Sephadex G-25 fine. The initial separation of the Folched material was carried out by chromatography on columns of DEAE-cellulose by performing successive elutions with various chloroform-methanol mixtures according to the Plackett method. The total glycolipids (CM 7.3) from each group were separated from phospholipids and isolated in large amounts by rechromatography on DEAE-cellulose. The chloroform-methanol (7:3) fraction contained most of the complement fixation reactive material. Subfractionation of the serologically active glycolipid fraction (CM 7:3) on silicic acid was carried out to purify the material for measurement of chemical and physical properties and for serological tests. Thin layer chromatography procedures have been described in previous reports.

Analytical Studies. The identification of the constituents in the serologically reactive glycolipid molecule for the purpose of classification of the molecule was carried out by hydrolysis of the purified material from the different groups of streptococci with $2N \cdot H_2SO_4$ for 2 hours at 110°C. Extraction of the hydrolysate with hexane separated the fatty acids from the aqueous phase which contained the glycerol and carbohydrate.

Major Findings:

Chromatography of the chloroform-methanol (2:1) lipid extracts from the different groups of streptococci studies on DEAE-cellulose separated the glycolipids of each group from the phospholipids. Thin layer chromatography of the glycolipid hapten (CM 7:3) fractions on borate-impregnated silica gel G plates showed very similar glycolipid spots when the plates were sprayed with periodate-Schiff reagent. As in Groups A and D streptococci, the B, C, E, G, and H group streptococcal glycolipids may have more than one glycolipid hapten. Analytical analysis of the purified serologically active hapten showed no detectable nitrogen or phosphorus. Some general information was derived from the hydrolysis of the active material as to the constituent moieties in the molecule. The fatty acid compositon of the CM 7:3 fraction showed a predominance of 16.0 and 18.0 carbon fatty acids, characterized as palmitic and stearic. Carbohydrate other than glycerol was detected as glucose in the CM (7:3) fractions from the groups of streptococci studied. The glycerol: fatty acid: carbohydrate ratio of the hapten preparation resembled those of diglucosyl diglyceride (2:2:2). Further fractionation of the serologically active chloroform-methanol 7:3 fractions was carried out by silicic acid chromatography. Thin layer chromatography of the nine fractions obtained

showed heterogeneity of each. The four serologically active subfractions consisted each of several glycolipids, as shown by their color reaction with the periodate-Schiff reagent and by carbohydrate analysis of hydrolysates.

Reaggregation. Since low level stimulation of complement-fixation activity with lipid haptens in rabbits is observed, experiments to reaggregate the serologically active streptococcal glycolipid (CM 7:3) with lipid-depleted membrane protein were carried out. The objective was to test the importance of membrane protein carrier in stimulating high complement-fixation activity for lipid hapten linked to it, in rabbits immunized with reaggregates. Reaggregation of streptococcal glycolipids with membrane proteins from Staphylococcus aureus or Acholeplasma laidlawii was not successful, as determined by electron microscopic examination for re-formed membrane. The method was that previously used successfully for reaggregating Mycoplasma pneumoniae glycolipid with A. laidlawii membrane protein. Preliminary studies with varying amounts of Mg^{2+} showed very little or no reaggregation of the lipid and membrane protein, and the proper combination of reaggregating conditions has yet to be found.

Antigenicity. The chloroform-methanol (7:3) lipid haptens from all groups of streptococci studied showed high titers in the complement-fixation test against sera of rabbits hyperimmunized with whole homologous streptococci. These same lipids cross-react with heterologous streptococcal rabbit antisera in the complement-fixation test and are serologically inactive in the complement fixation test with antisera to Staphylococcus aureus, Proteus mirabilis, Mycoplasma pneumoniae, and Pneumococcus Type I.

Deacetylated materials (i.e., the hexane soluble fraction and the water soluble fraction), after hydrolysis, were tested for complement-fixation activity. Neither the fatty acids nor the glycerol-carbohydrate fractions reacted in the complement-fixation test with rabbit hyperimmune serum singly or in mixtures.

Of the 9 samples from the subfractionation of the chloroform-methanol (7:3) serologically reactive glycolipid, one showed high complement-fixation activity with homologous and heterologous rabbit streptococcal hyperimmune sera, while mild complement-fixation activity was distributed in three other fractions. The remaining five fractions were serologically inactive.

Significance to Bio-Medical Research and the Program of the Institute:

The finding of easily-extractable, serologically cross-reactive, glycolipid haptens in a number of streptococcal groups, is of theoretical interest in terms of previously reported but undefined streptococcal membrane antigens of presumptive importance in graft-rejection reactions, rheumatic fever, and glomerulonephritis. Our studies provide as basis for determining the nature, specificity, and possible pathogenetic role of such suspect antigens, as well as for investigating relationships of molecular configuration to serologic activity. These studies will be aided by the distinct possibilities for synthesis of hapten, now that its nature is recognized as a relatively simple glycolipid of low molecular weight.

Proposed Course of Project:

Further work designed to define the chemical structure and molecular configuration of the serologically reactive glycolipid is in progress. Additional studies will be carried out on suitable means of reaggregating the streptococcal glycolipid hapten with lipid-depleted membrane proteins from Acholeplasma laidlawii, Staphylococcus aureus, and Streptococci as carriers, in order to clarify its antigenicity. Studies will also be performed on the biosynthesis of the serologically active glycolipid, using intact cells and cell-free preparations to determine the enzyme systems which catalyze the formation of precursors and the membrane glycolipid.

Honors and Awards:

None

Publications:

Caldes, G., and Prescott, B.: A comparison of the chemical compositon of Mycobacterium tuberculosis muris with Mycobacterium tuberculosis bovis (BCG). Biochem. Biophys. Res. Comm. 44: 852-858, 1971.

Serial No. NIAID 65
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electron microscopy of bacteria in relating structure and function.

Previous Serial Number: Same

Principal Investigator: Dr. Roger M. Cole

Other Investigators: Mr. Terry J. Popkin

Cooperating Units: University of Arizona, College of Liberal Arts, Dept. of Microbiology and Medical Technology, Tucson (Dr. Neil H. Mendelson, Dr. John N. Reeve)

State University of New York, Downstate Medical Center, Dept. of Medicine, Infectious Diseases Section, Brooklyn (Dr. Stephen J. Seligman)

University of Rochester, School of Medicine and Dentistry, Dept. of Microbiology (Dr. Anadi S. Chatterjee, Dr. Frank E. Young)

University of California, San Diego, Dept. of Biology (Dr. W. Claiborne Brown)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	21/12	-	-	21/12
Professional	18/12	-	-	18/12
Other	3/12	-	-	3/12

Project Description:

Objectives:

To examine the ultrastructure of microorganisms, in self-generated and collaborative studies, with particular emphasis on bacterial cell walls and membranes, their related antigens, and aspects of their participation in growth, division, genetic interchange, bacteriophage absorption, and the like.

Methods Employed:

Standard or newly-developed methods of bacteriology, serology, phage virology, microbial genetics, biochemistry, and electron microscopy. When appropriate, special methods of antibody labelling, cytochemistry, or other techniques of marking for electron microscopy.

(a) Bacillus subtilis mutants

1. The effects of a nitrosoquanidine-induced, temperature-sensitive, mutation in a strain subsequently called 168ts-200B, were previously described. In brief, at 45°C, the mutation results in loss of cell wall teichoic acid, loss of autolytic enzyme activity, conversion of normal rods to spherical forms growing in clusters of unseparated cells with random cross-walls, and grossly thickened cell walls with loss of periplasm and of wall layers. The mutant locus, which is now called tag-1 (teichoic acid glycerol) has been mapped on the B. subtilis chromosome, and by phage-mediated (PBS-1) transduction transferred by DNA-mediated transformation, to strains of different genetic backgrounds. We have examined the ultrastructural results of such locus transfer in 8 different strains or combinations. In all, the tag-1 mutation leads to the above-mentioned changes in cell wall ultrastructure--but, depending on the genetic constitution of the recipient in a fashion not yet understood, shape changes may range from complete conversion to sphere clusters, to chains of smaller spheres, to "dumb-bell" forms which are part sphere and part rod. Concomitantly, there occur variations in the random or normal placement of cross-walls at division, and the extent of separation of daughter cells after division. (Collaborators: Drs. Mendelson, Reeve, Young)

2. In similar fashion, the mutant locus of a salt-requiring strain, previously isolated as rod^{-}4 by Dr. H. J. Rogers (NIMR, Mill Hill, London), was transformed by him to strain BD54. We examined the ultrastructural results of this transfer in cells (now called rod 104) grown without salt. As in the original mutant under the conditions, rods become clusters of spheres which resemble tag-1 mutants in all ultrastructural respects except for the absence of periplasm. However, the rod^{-}4 mutation does not result in loss of wall, teichoic acid nor of autolytic activity under the restrictive conditions, and the locus on the B. subtilis chromosome is also quite different than that of the tag-1 locus.

3. We have re-examined 168ts-355, a filamentous, non-septate, temperature-sensitive mutant described last year, and a publication is in progress. Results of examination of cells treated with lysozyme at various stages of filamentation, in an effort to visualize the numbers of protoplasts and nucleoids, were inconclusive. Data on cell wall chemistry, phage adsorption, and genetic locus in this mutant are yet to be obtained. (Collaborator: Dr. Mendelson)

4. Autolysis of cells, which involves degradation of cell wall by native enzymes, is a common phenomenon among bacilli. The number of autolytic enzymes present is in some dispute, but at least two,and possibly three, have been noted in B. subtilis. These are characterized by different pH optima (6.0,

8.0, and 9.5), and must be demonstrated in strains which do not produce an autolysin-destroying protease. Since the possibility exists for different sites of activity for different enzymes, we attempted to localize such sites ultrastructurally by examining thin sections of bacilli sampled at intervals of time after suspension in a lysing buffer at each of the above pH's. The organism was B. subtilis SR22, an asporogenous, protease-negative mutant of strain 168. There were no differences seen at any pH. In all, there was very early lysis at the newly-forming cross-walls, which rapidly became marked, with rupture and loss of cell contents. However, a general erosion and thinning of the entire peripheral wall (except possibly at poles) also occurred early and resulted later in apparently random breaks in peripheral wall. The question of preservation of polar wall, which has been claimed by other investigators, is not clear; but our present results show no differences in sites of attack on cell wall when autolysis is carried out at three different pH optima. (Collaborator: Dr. W. C. Brown)

(b) Staphylococcus aureus mutants

1. A few additional strains of salt-dependent or methicillin-resistant staphylococci, grown under different conditions, were examined in thin section. As before, bizarre types of cell wall mutants were seen, often growing in clusters--but the relation of these changes to genetic composition, cultural conditions, or the presence or activity of autolysins, is totally unclear at present. (Collaborator: Dr. Seligman)

2. A known teichoic acid-deficient (TAD) mutant (52A5, or tar-1) of Staphylococcus aureus H (SM^R), was not previously described ultrastructurally. Thin sections showed that mutant cells are large, irregular in shape, have a thick irregular wall that is not trilaminar, and tend to grow in clusters of non-separating cells divided by randomly-placed cross-walls. The mutant thus resembles some TAD mutants of Bacillus subtilis. Parental (Staph. H) cells resemble other normal or wild-type staphylococci, having smaller, regular, separated cells with thin and regular tri-laminar walls. Results of varying conditions of growth or of incubation showed that walls of both parent and mutant became thicker and denser in overnight cultures or at 42°C, and those of the former lost trilaminarity and periplasm. This condition was corrected or prevented by the addition of 1 M NaCl, which was shown to cause (in growth media) a loosening of cell wall structure. This is attributed to a limited autolysis, because incubation of cells in buffer, in the absence of nutrients but in the presence of 1 M NaCl, led to gross autolysis as shown by optical density measurements and electron microscopy. Incubation in buffer without salt had no such effect. Autolysis was always most marked and rapid in the mutant, and in either parent or mutant cells when they had been initially grown in media containing 1 M NaCl. (Collaborators: Drs. Chatterjee, Young)

3. A series of temperature-sensitive mutants of Staphylococcus aureus H (str), which are defective in peptidoglycan synthesis at the restrictive temperature (42°C), were examined by thin sections of cells incubated at 30°C, 42°C, and 42°C in the presence of 1M NaCl. The presence of the latter corrects, chemically, the defects in peptidoglyan synthesis and permits growth at 42°C.

Ultrastructurally, the walls become more dense and compact at 42°C with a tendency to loss of trilaminarity, and this condition is prevented by NaCl. The effects thus resemble those seen in the parent strain (paragraph b, 2, above); and in the present stage of incomplete analysis, offer no ultrastructural findings uniquely attributable to defects in cell wall synthesis. (Collaborators: Drs. Chatterjee, Young)

4. Six mutants defective in production of autolytic enzyme have been examined in log phase growth. Two have normal cell wall. Three show increased density of wall with loss of trilaminarity, and thickening in two of these. One shows definite lysis of wall. Therefore, no consistent ultrastructural pattern related to lack of autolytic activity has been found, but the analysis is as yet preliminary. (Collaborators: Drs. Chatterjee, Young)

(c) Effects of salt on Staphylococcal ultrastructure

Because of varying effects of NaCl which were seen in the above-cited studies, we initiated an ultrastructural investigation of salt concentration effects on a known "wild type" strain of Staphylococcus aureus (ATCC 6538P). In either defined or complex media, increasing salt concentrations (up to 10.5%) led increasingly to larger cells, more loosely-structured walls of greater irregularity in thickness, evidence of lysis and of improved visualization of periplasm and mesosomes (at intermediate concentrations), and improved preservation of DNA strands with a trend toward compact or condensed nucleoids. The highest concentrations caused the greatest irregularities in cell shape, size, and wall thickness, and resulted in variation in thickness and placement of cross-walls. The effects on DNA were subject to rapid salt effects, since washing without salt prior to fixation resulted in immediate loss of compactness and preservation of nucleoids. (Mr. Popkin and Dr. Theodore)

(d) Electron microscopy in support of other projects

1. Lysates of Group H streptococci, following exposure to mitomycin C, were monitored for induction of bacteriophages. Five of 17 strains were lysogenic. The phages are morphologically identical with one another and with previously-described phages of Groups A and G streptococci. Stages in the intracellular development of one such phage, infecting a non-lysogenized strain of Group H, were followed by ultrathin sectioning. Development does not differ from that of other streptococcal phages. (Project No. NIAID 65A-1, Parsons)

2. The effects of cations (Mg^{++}), chelating agents (EDTA), delipidation methods, proteolytic enzymes, and phenol acetic acid extraction on the structure and integrity of isolated streptococcal membranes and mesosomes, was monitored by thin sectioning and negative staining techniques. (Project No. NIAID 63C, Schade)

3. Results of attempted reaggregation of streptococcal glycolipids with membrane proteins from other microorganisms were followed by thin sections. No re-formed "hybrid" membranes were detected. (Project No. NIAID 64D, Prescott)

4. Affirmation or denial of lysis in streptococci of 16 different serogroups by exposure to a lysin produced by genetically-competent Group H streptococci, was obtained by direct electron microscopy of negatively-stained material. Lysis occurred only in representatives of Group A, E, H, and K. In all instances, as in the original observations of autolysis of the competent streptococci, the lysis was restricted to the narrow equatorial band of newly-forming cell wall. A number of other Gram-positive bacteria were not lysed by the enzyme. (Project No. NIAID 65C-1, Ranhand)

5. Further sectioning of a new bacterium from nodules in an African snail was carried out. Attempted culture was unsuccessful. A morphologic and ultra-structural description is in progress, but data on routes and stages of infection, and on the taxonomic position of this Gram-negative microorganism, are lacking. (LPD, Project No. NIAID 121-C, Richards)

6. Miscellaneous and preliminary observations were made on presumptive mycoplasma viruses, and on presumptive mycoplasmas from plant diseases. (Project No. NIAID 205, Tully)

(e) Serological identification of "Group H" Streptococci

Following discovery of phages in 5 of 17 streptococcal strains supplied as"Group H" (Project No. NIAID 65A-1, Parsons) we wished to verify the serologic group of these strains, but found that the Center for Disease Control no longer supplies such grouping antiserum because of its inconsistency. We, therefore, initiated antiserum production in rabbits with some of our strains. Preliminary results show that two groupings occur among these strains: (1) carbohydrate extracts of three strains react with antiserum against a strain K208, and (2) extracts of all other strains, save one completely non-reactive, react with antisera against strain Blackburn. Antisera to several of these latter strains, or to a known strain of Streptococcus sanguis, behave like Blackburn antiserum. Of the three K208-reacting strains, 2 also cross-react with Blackburn antiserum, and one of these strains (F90A) is frequently used to produce "H" antisera. In England, strain Blackburn is used for antiserum production. In the U.S., strain K208 is also often designated as prototype Group H strain. Incomplete studies by precipitation analysis in immunodiffusion gels show two common and several variable antigens among Blackburn-reacting strains. Which is the group antigen, and which may represent type-specific polysaccharides, is not known, but work is being continued. It is likely that the confused situation in "Group H" serology arose because of some antigenic, but minor, determinants shared by some "classic" representatives of the two serologic groupings that we have found.

Significance to Bio-Medical Research and the Program of the Institute:

The purpose of our major studies at present is to clarify the relationships among bacterial growth and division, presence or absence of cell wall constituents, and cell wall autolytic enzymes. To this end, we are comparing ultrastructurally, various mutants of Gram-positive bacteria to one another and to normal or "wild-type" organisms--when grown under different conditions.

The results show that mutations, which can be specifically localized and transferred to a variety of genetic backgrounds in other strains,can effect production of specific cell wall components (for example, teichoic acid). Its lack, in turn, is associated with defects in autolytic activity and with concomitant ultrastructural changes in cell size, shape, division, and nature of the cell wall--in both Bacillus and Staphylococcus. Conditions which stimulate or inhibit autolytic activity have been shown to produce characteristic cell wall, other morphologic, and growth, changes--even in wild-type bacteria, although to a lesser extent than in mutants. We are, therefore, approaching definitive answers to the genetic basis for, and intermediary regulatory factors in, the control of growth and division among walled microorganisms which normally maintain a characteristic size and shape.

Proposed Course of Project:

Studies of normal and mutant bacteria, and of isolated bacterial components, to be continued. Further investigation of "Group H" streptococcal serology is in progress.

Honors and Awards:

Invited participant, Gordon Conference on "Bacterial Cell Surfaces," Holderness School, N. H., July 1971.

Presented seminar on "Bacterial Cell Wall Mutants," Dept. of Microbiology, University of Rochester (N.Y.) School of Medicine and Dentistry, Oct. 1, 1971.

Invited principal speaker on "Structure and Function of Bacterial Cell Surfaces," meeting of the Central New York Branch, American Society for Microbiology at Rochester (N.Y.) Institute of Technology, Oct. 2, 1971.

Presented seminar, "Bacterial Cell Surfaces," Dept. of Biology, Catholic University, Washington, D. C., Dec. 6, 1971.

Presented seminar, "Problems of Ultrastructure of Bacterial L Forms," Armed Forces Institute of Pathology, Cell Biology and Electron Microscopy Section, Washington, D. C., April 11, 1972.

Invited participant, Seminar on Bacterial L Forms, Annual Meeting, American Society for Microbiology, Philadelphia, April 23-28, 1972. Chairman, two scientific sessions at same meeting.

Awarded Meritorious Service Medal, U.S.P.H.S. (Cole)

Library Advisory Committee, NIH

Project Officer, NIAID Research Contract PH 43-68-83, University of Chicago.

Convener, Workshop on Streptococcal M Protein, Collaborative Research Branch, NIAID, November 3,4, 1971.

Cole, R. M.: Some implications of the comparative ultrastructure of bacterial L forms. In. Madoff, S. (Ed.): Mycoplasma and the L Forms of Bacteria. Gordon and Breach Publishing Co., New York, N. Y., 1971, pp.49-83.

Ranhand, J.M., Leonard, C. G., and Cole, R. M.: A comparison of streptococcal competence factors produced by strain Challis in chemically defined and complex media. J. Gen. Microbiol. 65: 131-138, 1971.

Ranhand, J.M., and Cole, R. M.: Demonstration of transforming DNA in an autolysate of a group H streptococcus. J. Bacteriol. 106: 712-713, 1971.

Colon, A. E., Leonard, C. G., and Cole, R. M.: Lysis and lysogenization of Groups A, C, and G streptococci by a transducing bacteriophage induced from a Group G streptococcus. J. Virology 8: 103-110, 1971.

Theodore, T. S., Popkin, T. J., and Cole, R. M.: The separation and isolation of plasma membranes and mesosomal vesicles from Staphylococcus aureus. Prep. Biochem. 1:233-247, 1971.

Popkin, T. J., Theodore, T. S., and Cole, R. M.: Electron microscopy during release and purification of mesosomal vesicles and protoplast membranes from Staphylococcus aureus. J. Bacteriol. 107: 907-917, 1971.

Glick, A. D., Getnick, R. A., and Cole, R. M.: Electron microscopy of group A streptococci after phagocytosis by human monocytes. Infection and Immunity 4: 772-779, 1971.

Ranhand, J. M., and Cole, R. M.: Lysis of streptococci by an extracellular lysin produced by competent Group H streptococcus strain Challis. J. Gen. Microbiol. 70: 1972.

Colon, A. E., Cole, R. M., and Leonard, C. G.: Intergroup lysis and transduction by streptococcal bacteriophages. J. Virology 9: 551-553, 1972.

Leonard, C. G., and Cole, R. M.: Purification and properties of streptococcal competence factor isolated from chemically defined medium. J. Bacteriol. 110: 273-280, 1972.

Parsons, C. L., Colon, A- E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from "Group H" streptococci. J. Virol. (in press)

Serial No. NIAID 65A
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Genetics of Group A Streptococci

Previous Serial Number: Same

Principal Investigator: Alba E. Colon

Other Investigators: Carmen G. Leonard
C. Lowell Parsons
Unnur Petursdottir (Guest Worker)
Roger M. Cole
Robert M. Cohen (Guest Worker)

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	18/12	-	3/12	21/12
Professional	15/12	-	1/12	16/12
Other	3/12	-	2/12	5/12

Project Description:

Objectives:

To continue studies of bacteriophage-mediated transduction within and between several streptococcal groups, using antigenic as well as antibiotic markers. To investigate streptophage specificity and lysogenic conversion.

Methods Employed:

Last year we reported intergorup lysis of two Group C strains by a Group G temperate phage (GT234), a Group A temperate phage (AT298) and a Group A virulent phage (A25). Further studies were conducted to determine if these known transducing phages, as well as others induced from streptococci of other serological groups, were capable of intergroup lysis and transduction. A total of 191 streptococcal strains, representing Groups A, B, C, D, F, E, H,

K, L, M, N, O, and P, were tested by the agar surface method for lysis by 8 different streptococcal phages. Bacteriophages containing lysates from streptococci of Groups A, E, and C lysed streptococci distributed among serogroups A, C, G, H, and L. The phages plaqued and propagated to high titers in Groups A and C but not in Groups G, H, and L. Agar-surface lysis did not occur with phages neutralized by their specific antisera, or with phage-free (centrifuged) supernatants. However, lysis did occur at a 1:1 PFU/CFU ratio, and with crude phage-containing supernatants heated to 45°C for 15 min to inactivate phage-associated lysin. Therefore, lysis from without or by a free enzyme was ruled out. The results suggest an infectious cycle releasing altered phage progeny that were mutated, defective or otherwise unable to propagate in the same strain.

Following the demonstration of intergroup lysis, we examined 123 phage sensitive strains of Groups A, C, G, H, and L, as possible recipients of transducing DNA. The streptomycin resistance marker was used. Group A virulent phage (A25) and Group A temperate phage (AT298), that transduce within Group A, were also found capable of transducing streptomycin resistance to one strain of Group G. Furthermore, temperate phages from Groups E and G, when propagated in the Group A donor, transduced the marker to other Group A strains.

Significance to Bio-Medical Research and the Program of the Institute:

Intergroup lysis and intergroup transduction of the streptomycin marker between streptococcal groups have been demonstrated. These results present the possibility of similar intergroup transduction of other markers such as M protein, wall carbohydrate, etc. There is also indicated a need for reassessment of phage group specificity and receptor sites, as well as further studies on the phage-host relationship among streptococcal groups. A clearer knowledge of streptococcal bacteriophage and their role in phage-host relationship and bacterial genetic exchange is important to further understanding of streptococci, their epidemiology, and their role in disease.

Proposed Course of Project:

The possibilities of lysogenic conversion to erythrogenic toxin production, by lysogenizing Group C streptococci with appropriate Group A phages, are now in progress. Studies are also being conducted to find a transducing phage for Group H streptococci. It is further planned to study the possible genetic exchange of antigens between streptococcal groups and within Group A streptococci.

Honors and Awards:

None

Publications:

Colon, A. E., Cole, R. M., and Leonard, C. G.: Intergroup lysis and transduction by streptococcal bacteriophages. J. Virol. 9: 551-553, 1972.

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from Group H Streptococci. J. Virol. (in press)

Serial No. NIAID 65A-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Group H streptococcal bacteriophages: isolation and effects on genetic systems of transformable streptococci.

Previous Serial Number: None

Principal Investigator: Dr. C. Lowell Parsons

Other Investigators: Dr. Roger M. Cole
Miss Alba E. Colon
Mrs. Carmen G. Leonard
Dr. Jon M. Ranhand

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	17/12	-	-	17/12
Professional	17/12	-	-	17/12
Other	0/12	-	-	0/12

Project Description:

Objectives:

To isolate and characterize bacteriophages capable of infecting and lysogenizing transformable strains of streptococci (serologically, members of Group H), in order to explore the relationships of phage-mediated transduction, phage DNA-mediated transfection, and lysogeny to genetic competence.

Methods Employed:

Standard methods of bacteriological culture and bacteriophage assay. Mitomycin C was used to induce vegetative phage propagation in lysogenic bacteria.

Major Findings:

By mitomycin C treatment, 5 of 17 strains of streptococci identified as Group H were found to be lysogenic. Two of the phages isolated were shown to infect, and also to lysogenize, the highly-transformable and non-lysogenic strains Challis and Wicky. The phage DNA was capable of transfecting competent cultures of these strains, to produce infectious phage progeny. Furthermore, the lysogenized cultures, cloned and passed in the presence of phage antiserum, were found markedly inhibited in transformability. Nevertheless, such cultures demonstrated an autolytic phenomenon previously associated only with competent cells; and lysogenized Challis continued to produce competence factor. Experiments with radioactively-labelled DNA demonstrated that the lysogenized cells were no longer capable of binding DNA. These results indicate that late, but not early, stages of competence development can be affected by the presence of a prophage.

Significance to Bio-Medical Research and the Program of the Institute:

Bacteriophages for Group H streptococci were previously unknown. Their discovery, demonstration of transfection with their DNA, and the probability of their future use in transduction, now make possible a greater variety of approaches to the nature of competence and to problems of genetic exchange. Similar systems are known in other bacteria, but have never before been available for streptococci. The surprising effect of lysogeny on transformation efficiency, though reported once in Bacillus subtilis, is a relatively new phenomenon for which the explanation at a molecular level is unknown. Its lack of effect on autolysis, but later prevention of DNA binding, has enabled a clearer understanding of the sequence and separability of those events which, initially set in progress by competence factor, lead to genetic transformation.

Proposed Course of Project:

We plan to employ transfection for studies of competence, and to develop a system of transduction for further aid in clarifying bacterial genetic mechanisms in general, and those of streptococci in particular. Further study of the lysogenic state--both to elucidate stages of competence and to discover the reasons for the failure of DNA binding--are currently in progress. Methods of curing lysogeny are planned, to determine if its inhibitory effects on transformation are reversible.

Honors and Awards:

Invited participant, XV Annual Meeting on Microbial Transformation, Estes Park, Colorado, June, 1972. (Parsons)

Publications:

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolation of bacteriophages from Group H streptococci. J. Virology 9: , 1972.

Serial No. NIAID 65B
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the identification of bacterial L forms and Mycoplasma species.

Previous Serial Number: Same

Principal Investigator: Theodore S. Theodore and Joseph G. Tully

Other Investigators: Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1971 Annual Report.

Research project terminated. Capabilities will be maintained only for service for the routine identification of bacterial L forms and Mycoplasma species, as required.

Honors and Awards:

Invited to present lecture and conduct a workshop on "The Use of Polyacrylamide Gel Electrophoresis for the Identification of Bacteria," at the Henry Ford Hospital, Dept. of Ophthalmology, Detroit, Michigan on April 18 and 19, 1972. (Theodore)

Publications:

None

Serial No. NIAID 65C
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Competence development and genetic transformation in Group H streptococci: Nature and role of competence factor

Previous Serial Number: Same

Principal Investigator: Mrs. Carmen G. Leonard

Other Investigators: Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	33/12	-	-	33/12
Professional	24/12	-	-	24/12
Other	9/12	-	-	9/12

Project Description:

Objectives:

To define the competent state as it develops in Group H streptococci and to determine the chemical nature and mode of action of competence inducing factor(s) (CF).

Methods Employed:

The usual methods in microbial genetics, and standard biochemical methods, were used. We developed procedures for the isolation and purification of competence factors by modifications of known techniques of column and thin layer chromatography, electrophoresis, and isoelectric focusing.

Major Findings:

A. Studies on the chemical nature of competence factor (CF):

The extremely small amounts of CF produced by Challis cells, its apparent instability, and the strong tendency of CF to absorb to some proteins, glass,

cellulose, sephadex, acrylamide gel and other chromatographic materials, have long hampered its purification and identification. Nevertheless, we have obtained sufficient amounts of highly purified CF, by further improvements in the purification procedures described last year, which is of very high purity. This report and a recent publication present the first extensive characterization and chemical analysis of highly purified CF. It is a small, dialyzable, thermoresistant compound which is free of lipids, phosphorus, and carbohydrates. It is composed of 90% protein (as determined by the Lowry reaction) containing 15% organic nitrogen and is a positively charged, basic compound with an extremely high isoelectric point of above pH 11.0. The fact that the purified CF has such a high isoelectric point, that it is sensitive to trypsin, that it is precipitable by TCA, and that it lacks detectable amounts of sulfur-containing amino acids, suggests that it may be a protamine or protamine-like compound rich in arginine or lysine, or both. CF is unlikely to be a free polyamine, since it is precipitated by TCA and is sensitive to trypsin. However, a polyamine could be a part of a larger macromolecule, with a peptide acting as a carrier for the polyamine. Further chemical analyses of CF obtained after isoelectric focusing are in progress. The extremely basic nature of CF suggests several possible roles, such as altering the negative bacteria surface charge as well as affecting other processes required to bind the highly acidic DNA at the membrane level.

B. Studies on the initial steps in competence development:

In an earlier publication we showed conclusively that CF is produced prior to competence development, and that CF production and competence development are separable and independent events. By appropriately timed addition to competence-supporting media of proteolytic enzymes--which destroy released CF but do not affect cells already competent--we have now shown the sequence of events in competence development to be: first, release of CF from cells (strain Challis) which contain cell-bound CF from the outset but are not initially competent; second, binding of this released or exogenous CF by the producing cells (Challis) after washing, or by added cells of a strain (Wicky) which does not make CF; third, induction of those incompletely-understood steps measured by appearance of new proteins and autolytic phenomenon (Project No. NIAID 65C-1, Ranhand), which finally result in DNA uptake and recombination. All of these latter comprise the development of the competent state by the newly-bound CF. At step 2, the cells must be at a specific physiological age. In step 3, induction of competence occurs only if protein synthesis proceeds uninterruptedly for 40 minutes after CF is added. The important conclusion is that only reabsorption of exogenous CF (in a complete medium) leads to competence development, even in cells which produce CF and continually retain it in a cell-bound form.

Significance to Bio-Medical Research and the Program of the Institute:

We have obtained the first comprehensive characterization and chemical analysis of streptococcal competence factor. This information has enabled us to postulate possible roles and mechanisms of induction of the competent state by CF, including the possible role of CF in the reversible and irreversible

binding of DNA by competent cells. The availability of characterized CF now permits direct testing of these postulates. In addition the improved method developed for the isolation and purification of extremely small amounts of this highly charged bacterial product will be of value in the isolation of other such microbial products.

Proposed Course of Project:

We plan further characterization of the chemical nature of CF--particula its amino acid composition--after purification by isoelectric focusing. Its effects will be explored by study of the newly-induced proteins and of possib changes in bacterial surface charge. The nature of its initial binding will be further explored by use of inhibitors such as antibiotics, proteolytic enz or other hydrolysing agents. Included will be comparisons of various attribu of lysogenic versus non-lysogenic cultures of Group H streptococci, in which lysogeny has been shown to alter transformability. (See Project No. NIAID 65 Parsons)

Honors and Awards:

None

Publications:

Leonard, C. G., and Cole, R. M.: Purification and properties of strepto coccal competence factor isolated from chemically defined medium. J. Bacteri 110: 273-280, 1972.

Parsons, C. L., Colon, A. E., Leonard, C. G., and Cole, R. M.: Isolatio of bacteriophages from "Group H" streptococci. J. Virol. (in press)

Colon, A. E., Cole, R. M., and Leonard, C. G.: Intergroup lysis and transduction by streptococcal bacteriophages. J. Virol. 9: 551-553, 1972.

Colon, A. E., Leonard, C. G., and Cole, R. M.: Lysis and lysogenization of Groups A, C, and G streptococci by a transducing bacteriophage induced from a Group G streptococcus. J. Virol. 8: 103-110, 1971.

Serial No. NIAID 65C-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Competence development and genetic transformation in Group H streptococci: Nature and role of autolysins

Previous Serial Number: None

Principal Investigator: Dr. Jon M. Ranhand

Other Investigators: Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	-	-	24/12
Professional	12/12	-	-	12/12
Other	12/12	-	-	12/12

Project Description:

Objectives:

Our objective is to define the role that autolysins have in the development of competence in Group H streptococci. Competence is the ability of bacterial cells to react with exogenously supplied deoxyribonucleic acid (DNA) and thereby become genetically changed, or transformed. The nature of competence is not fully understood in streptococci nor in any of the other known transformable bacteria, i.e., pneumococci, Haemophilus. We have presented evidence that competence development involves an autolytic enzyme(s).

Methods Employed:

Standard bacterial methods are used. Competence is a function of the cell growth cycle and occurs spontaneously in some strains of streptococci and is induced in other strains by published methods. Autolysis of competent cultures is measured as a decline in optical density when such cells are suspended in a buffer consisting of Tris (hydroxy methyl) methyl-amino-propane sulfonic acid containing 2-mercaptoethanol and EDTA at pH 9.

Major Findings:

Last year we reported some new and major findings that we believe yield an understanding of the nature of competence in Group H streptococci. We demonstrated the presence of (a) a protein that seems specific to competent cells, and (b) a lytic system that is also restricted to competent cells. T function of the protein is not yet realized. However, the lytic phenomenon allows one to postulate a mechanism on how DNA can reach the cell membrane. Local, limited autolysis removes specific regions of the cell wall and there allows the DNA to approach specific uptake sites. Whether the autolysin and new protein are one and the same remains to be elucidated.

In the past year, I studied the specificity of the competent cell autol More specifically, I studied the soluble lytic factor that is produced by competent Challis cells for its lytic range, by using as substrate whole organisms from other bacterial genera as well as other groups of streptococc Of the 16 serological groups of streptococci tested, only strains from groups A, E, H, and K were lysed. Strains from Groups B, C, D, F, G, K, K, M, N, P, Q, R, and S were refractory. A minimum of two strains from each group was tested, except for Group E where only one strain was available. Only one strain from Group K lysed, whereas another did not. This observation points out probable differences in cell wall composition of cells that are related serologically. Other Gram positive bacteria were not lysed by the enzyme. These comprised cells from (a) Bacillus subtilis, (b) Micrococcus lysodeiktic (c) Staphylococcus aureus, and (d) Lactobacillus acidophilus.

Significance to Bio-Medical Research and the Program of the Institute:

The nature of competence (the ability of cells to take up DNA) is still a poorly understood phenomenon. The Group H streptococci produce a soluble factor (CF) that induces the competent state in otherwise noncompetent cells, and therefore, the degree or extent of competence as it exists in a populatio of cells can be controlled. Since we can experimentally control the level of competence, we can determine the events that lead to its full attainment. Most of the above reactions can be carried out in defined media. The new competence associated protein as well as the autolytic system recently descril supply useful new criteria in determining the site and mode of action of CF. With these criteria as tools, the biochemical and physiological changes that occur in cells that are becoming competent can be further studied.

Proposed Course of Project:

We plan to investigate in detail the nature and location of the newly synthesized competent cell associated protein. We also want to establish the role played by the autolysin in competence development. To date, accumula data suggests that there is a direct correlation between the cells ability to autolyze and its ability to develop competence. We will also attempt purifi- cation of the autolysin since it has a very unique mode of action; it attacks only newly synthesized cross wall.

Honors and Awards:

Session Chairman and invited participant, XVth and XVIth Annual Meeting on Microbial Transformation, Estes Park, Colorado; June 1971 and 1972.

Publications:

Ranhand, J. M., and Cole, R. M.: Demonstration of transforming DNA in an autolysate of a Group H streptococcus. J. Bacteriol. 106: 712-713, 1971.

Ranhand, J. M., Leonard, C. G., and Cole, R. M.: A comparison of streptococcal competence factors produced by strain Challis in chemically defined and complex media. J. Gen. Microbiol. 65: 131-138, 1971.

Ranhand, J. M., and Cole, R. M.: Lysis of streptococci by an extracellular lysin produced by competent Group H streptococcus strain Challis. J. Gen. Microbiol. 70: (in press)

Serial No. NIAID 65F
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The cell wall constituents found in a stabilized L form

Previous Serial Number: Same

Principal Investigators: James R. King and Benjamin Prescott

Other Investigators: George Caldes

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	7/12	-	-	7/12
Professional	6/12	-	-	6/12
Other	1/12	-	-	1/12

Project Description:

Objectives:

To determine the presence of, and to characterize, any cell wall constituents in a stable L form of a Group D streptococcus (strain F24).

Methods Employed:

All purification and analytical procedures are modifications of previously existing methods. Complex and lengthy methods of column chromatography, paper chromatography, colorimetry, and chemical extraction were used. The major operations are indicated below.

Polysaccharides were extracted from washed parent strain organisms or L forms by each of the following reagents: (1) sodium hydroxide, and (2) phenol. Selective precipitations and dialyses were used to remove exogenous contaminants introduced to solubilize polysaccharides. Purified polysaccharide was analyzed for monosaccharides, particularly rhamnose (an indicator of cell wall). In addition, general quality controls for extraneous materials were carried out on

all finished products.

Major Findings:

In previous years, serological comparisons of alkali-extracted polysaccharides from Streptococcus faecium F 24 parent strain and its L form showed no common antigenic determinants. The L form polysaccharide could be obtained only in very low yields, and the extracts contained contaminant with high nitrogen, low phosphorus, and high ash content. The reason for and the nature of this artifact is unknown. This year, we reported that more efficient alkali extracts ion and purification procedures produced virtually no demonstrable L form polysaccharide as determined by gorurrmetric analysis. Neither did an alternate phenol extraction method produce demonstrable polysaccharide. On the other hand, either purification and extraction method produced very pure polysaccharides from the parent strain. We conclude that F24L has no polysaccharide. This report completes a survey of the L form for cell wall material known to be in parent strain cell wall.

Significance to Bio-Medical Research and the Program of the Institute:

Our investigations confirm, chemically, the lack of cell wall or its components in the stable F24L. Other studies have shown, or not shown, wall components on several different L forms. The potential importance of these diverse findings is related to reported killing of several L forms - but not th r parent bacteria - by a complement-dependent antibody of normal human serum. It was suggested that cell wall, in the parent, prevents the bactericidal effect of what is anti-membrane antibody. While electron microscopy of the L forms showed no wall, no chemical search for wall components was made. It cannot be said with any certainty, therefore, that antibody directed against some wall determinants could not also participate in the serum killing effect. This is a point of interest because growth-inhibiting (?killing) anti-L form antibodies induced by active immunization of animals, are definitely directed against membrane antigens (see Project No. NIAID 85I 1970-71). F24L now makes possible a model system for testing the question in human sera, since this L form has been exhaustively studied for several years by several methods, and none indicate the presence of polysaccharides that characterize the cell wall of the parental bacterium.

Proposed Course of the Project:

With the current demonstration of the absence of cell wall constituents from Streptococcus faecium F24 L form, these chemical studies are now complete. The project will be terminated, pending completion of publications from this work.

Honors and Awards:

None.

Publications:

None.

Serial No. NIAID 65G
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Characterization of biophysical and physiological properties of L forms

Previous Serial Number: Same

Principal Investigator: James R. King

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	10/12	-	-	10/12
Professional	10/12	-	-	10/12
Other	-	-	-	-

Project Description:

Objectives:

To define the biophysical properties of L forms of bacteria; and to study the physiological response of L forms to *in vivo* environments.

Methods Employed:

Quantitative bacteriological techniques, such as statistically valid bacteriological plate counts, quantitation of bacterial mass by turbidimetric methods; controlled use of known metabolic inhibitors to determine metabolic pathways.

Major Findings:

(A) An improved light microscopic technique for examination of L forms was reported last year. The technique was intended for quantitiative use.

However, further experimentation revealed that the technique could not be used for quantitative purposes, because an unpredictable number of L forms could not be recovered by the mounting method. On the other hand, the preliminary observations are still valid concerning the usefulness of this method for ease of rapid mounting, removal of artifacts, and staining of material.

(B) Killing of Group A streptococcal L forms (strain AED-L) by simple centrifugation has been reported by other workers. Last year, I reported the lack of killing of enterococcal L forms (strain F24L) by centrifugation. In both of the above studies only one magnitude of relative centrifugal force (RCF), a function of speed was used. In the current reporting period, the RCF was varied and the type of centrifuge rotor was varied (angle rotor vs. horizontal rotor). Viability of AED-L or F24L remained constant in samples centrifuged with either rotor at any RCF tested. To insure statistical validity, ten replicate plates were inoculated for each RCF (ranging from less than 1000 to 12,000 X g). The parameter of time (0-30 minutes) was examined, and there was no killing of L forms. Centrifugation did not kill Group A or D streptococcal L forms, contrary to reports by other investigators.

(C) Other investigators reported that a normal human antibody-complement system caused very rapid killing of several strains of bacterial L forms. Those findings have been corroborated in this laboratory and extended to a study of the effect of normal serum on the viability of L forms coated with spermine, a substance known to protect bacterial protoplasts from osmotic lysis. In some experiments the spermine-grown L forms exhibited resistance to the lethal action of normal serum. This killing effect was also seen in horse serum, fetal bovine serum, and human plasma. Gamma-globulin free horse serum was used as a control, and no killing was observed.

(D) During the course of experiments on the reversion of Group A streptococcal L forms, Group A parent strain GL8 was treated overnight with penicillin (1000 units per ml) in broth containing salt (0-3.0%). The organisms were completely susceptible to penicillin at 0% salt (growth conditions) and completely resistant to penicillin in 3% salt (non-growth conditions). This was tested by degrading the penicillin by penicillinase and subsequent plating of the survivors in the absence of an osmotic stabilizer. The percentage of dead organisms correlated with the growth rate at any salt concentration in the absence of penicillin. The same experiment was done with a salt-tolerant mutant of GL8, and it was killed by the penicillin at all salt concentrations tested. The antagonism between salt and penicillin may mean that Group A streptococci of L forms and eventually reappear as apparent revertants.

Significance to Bio-Medical Research and the Program of the Institute:

In order to assess the role of any microorganism in disease, accurate and precise information must be obtained about its physical and physiological properties. The biomedical literature abounds in erroneous conclusions about L forms, which might affect clinical practice adversely. For example, an informed clinician would not centrifuge a specimen of fluid to concentrate suspected L forms because of reported lethal effects. The results of this report show conclusively that centrifugation does not affect viability of two strains

of streptococcal L forms. A clinician might be concerned about the likelihood of reversion of L forms to the parent strain. Apparent reversion of penicillin-induced Group A streptococcal L forms has been reported for two decades, but the resistance of Group A streptococci to penicillin at high salt concentrations suggests that apparent reversion may be the outgrowth of contaminating streptococci rather than reversion.

Toward more immediate concerns, I have shown that L forms are killed in vitro by by natural antibodies from several animal sources. However, this innate immunity can be partially blocked by spermine attached to the surface of tested L forms. For the most part, these observations corroborate and extend the studies by other investigators, who concluded that L forms have no role in infectious diseases.

Proposed Course of Project:

The doubtful relationship of L forms to most infectious diseases realistically requires that this project on L forms should be terminated upon completion and publications of experiments currently under way.

Honors and Awards:

None

Publications:

None

Serial No. NIAID 65H
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Evidence for the Degradation of Streptococcal Cell Walls by Human Polymorphonuclear Leukocytes.

Previous Serial Number: Same

Principal Investigator: Dr. Richard A. Getnick

Other Investigators: Dr. Alan D. Glick
Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1971 Annual Report.

Project terminated.

Serial No. NIAID 65H-1
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the fate of Group A streptococci phagocytized by human monocytes and polymorphonuclear leukocytes.

Previous Serial Number: Same

Principal Investigators: Dr. Alan D. Glick

Other Investigators: Dr. Jon M. Ranhand and Dr. Roger M. Cole

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	15/12	-	-	15/12
Professional	15/12	-	-	15/12
Other	-	-	-	-

Project Description:

Objectives:

To determine the fate of the mucopeptide portion of the Group A streptococcal cell wall after phagocytosis by human leukocytes, with particular emphasis on the factors influencing the susceptibility of the mucopeptide to enzymatic degradation.

Methods Employed:

Group A Streptococcus pyogenes (an M-negative strain derived from Lancefield strain T1/155) was grown in Brain Heart Infusion broth. Cell walls were isolated by shaking the organisms with 66μ diameter glass beads in a Braun disintegrator. Cell wall polysaccharide was removed by the use of hot formamide or dilute hydrochloric acid. Chemical modification of the mucopeptide was achieved by treatments with acetic anhydride and sodium hydroxide.

For the preparation of leukocyte granule extract, human leukocytes were suspended in 0.34 M sucrose and disrupted by sonic treatment. The lysosomal granules were isolated by differential centrifugation, and disrupted by repeated freezing and thawing to create an enzyme extract. Egg-white lysozyme was obtained from Worthington Biochemical Corporation. The activities of the enzyme preparations were monitored by assay against killed Micrococcus lysodeikticus.

Turbidometric studies and electron microscopy were done according to standard methods. To study phagocytosis, streptococcal mucopeptide shells were added to heparinized human blood and incubated at 37°C with constant shaking. Buffy coat preparations were fixed for electron microscopic observation.

Major Findings:

Last year we reported that streptococci, ingested by human monocytes, retained undergraded cell walls in spite of the loss of cytoplasmic contents. Human polymorphonuclear leukocytes were only able to dispose of a limited amount of cell wall material.

Group A cell walls are resistant to egg-white lysozyme. This has usually been assumed to be due to a protective effect conferred by the presence of cell wall polysaccharide. However, removal of most of this polysaccharide resulted in a minimal increase in sensitivity to lysozyme or leukocyte lysosomal enzymes as tested by turbidometry and electron microscopy. This indicated that other factors must be considered to be responsible for the resistance of the cell wall to enzymatic degradation. The Group A cell wall mucopeptide contains a large number of free (unsubstituted) amino groups which are known to inhibit lysozyme action. The acylation of these free amino groups was found to be necessary for optimal degradation by lysozyme or human leukocytes. However, the procedures used for acylation also resulted in acylating free hydroxyl groups in the mucopeptide. Because these O-acyl groups are also known to inhibit lysozyme activity, it was necessary to remove them before complete wall degradation could be obtained.

These results indicate that the factors responsible for the lysozyme-resistance of Group A cell walls also influence their resistance to degradation by human lysosomal enzymes and suggest that the chemical nature of the mucopeptide, in addition to the presence of cell wall polysaccharide, determines this resistance.

Significance To Bio-Medical Research and the Program of the Institute:

Group A cell walls or their components are known to persist in animal tissues and are associated with chronic inflammatory lesions. Theoretically, these cell walls may also play a role in post-streptococcal sequelae in man. Our studies have shown that the cell wall is resistant to degradation by human leukocytes, which appears to be the result of the cell wall polysaccharide and free amino groups in the mucopeptide. Therefore, the Group A cell wall

may persist in human leukocytes in vivo, and may be sequestered in those tissues where phagocytes localize. This cell wall material may produce or perpetuate chronic inflammatory lesions as occurs in experimental animals.

It remains, however, to demonstrate that the Group A cell wall induces lesions of any kind in man.

Proposed Course of Project:

If tissues become available soon, human rheumatic lesions will be examined by electron microscopy for the presence of residual Group A streptococcal cell walls. However, the principal investigator will complete his Staff Associateship in June 1972, and the project will probably terminate at that time--or until new personnel with appropriate interests and skills are obtained.

Honors and Awards:

None

Publications:

Glick, A. D., Getnick, R. A., and Cole, R. M.: Electron microscopy of Group A streptococci after phagocytosis by human monocytes. Infection and Immunity 4: 772-779, 1971.

Serial No. NIAID 65I
1. Microbiology
2. Bacterial Structure and Function
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunology of Streptococcal L Forms

Previous Serial Number: Same

Principal Investigator: Dr. Susan E. Birnbaum

Other Investigators: Dr. Roger M. Cole
Dr. Benjamin Prescott

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	-	-	-	-
Professional	-	-	-	-
Other	-	-	-	-

Project Description:

See 1971 Annual Report.

Project terminated.

Serial No. NIAID 77-1-C
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ecologic and Aerobiologic Studies of Histoplasma capsulatum

Previous Serial Number: Same

Principal Investigator: K. J. Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	0/12	-	-	0/12
Professional	-	-	-	-
Other	-	-	-	-

Project Description: Project incorporated with NIAID 79C-1.

Serial No. NIAID 77-1-F
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Aspergillus bisporus

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	5/12	-	-	5/12
Professional	2/12	-	-	2/12
Other	3/12	-	-	3/12

Project Description:

Objectives:

To determine the effect of osmolarity of urine on the pathogenicity of A. bisporus in mice.

Methods Employed:

Fifty two female STR/N mice (Inbred mice with primary polydipsia) and general purpose (G.P.) female mice were injected with 2 million spores of A. bisporus intravenously. The two groups included subgroups (13 each) as follows: 1) Group A, STR/N mice with normal consumption of water, 2) Group B, STR/N mice with restricted water consumption (one third of normal consumption), 3) Group C, G.P. mice with normal intake of water, 4) Group D, G.P. mice provided with 5% sucrose solution to enhance the liquid consumption. The urine was collected daily from each group and its osmolarity measured. The daily water consumption by each group was also compared. All mice were sacrificed five weeks after injection. A portion of the kidneys with signs of abnormality were fixed and prepared for histological sections and the remainder used for the recovery

of the fungus.

Major Findings:

Group	Average daily water intake/mouse	Average urine osmolarity	Number infected/ Number injected
(A) STR/N	16.2 ml	369	0/13
(B) STR/N (1/3 H_2O)	5.8	806	3/13
(C) G.P.	6.2	617	3/13
(D) G.P. (5% sucrose)	14.1	564	2/13

The results show that mouse strain or average daily water intake were unrelated to occurrence of renal infection by A. bisporus. Although the numbers are small, they suggest that the influential factor was urine osmolarity. If this was maintained, by different means, at or above the level normal for G.P. mice, infection occurred.

Significance to Biomedical Research and the Program of the Institute:

Aspergillus bisporus is a recently described pathogen of mice which multiplies only on the lumen of the extreme distal ends of the collecting tubules and in the pelvis of the kidney. It is also unique in its ability to grow on media containing 40% sucrose. However, the apparent influence of urine osmolarity on its growth in vivo may have application in study of other fungi, or other microorganisms causing renal infections of man or animals.

Proposed Course of the Project:

The project will be terminated.

Honors and Awards:

None.

Publications:

Kwon-Chung, K. J., Fennell, D. I.: A new pathogenic species of Aspergillus. Mycologia 63: 478-489, 1971.

Serial No. NIAID 77-1-G
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetic study of pathogenic fungi

Previous Serial Number: Same

Principal Investigator: Kyung Joo Kwon-Chung

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	23/12	-	-	23/12
Professional	7/12	-	-	7/12
Other	16/12	-	-	16/12

Project Description:

Objectives:

A) To investigate the relationship between virulence, pigment of cultures and capability of mating in Ajellomyces dermatitidis (Blastomyces dermatitidis), and B) to detect the presence of the sexual state in Histoplasma capsulatum and geographic distribution of the two mating.

Methods Employed:

A) Ten of the most sexually active F_1 and F_2 progeny of Ajellomyces dermatitidis (ATCC 18187 x ATCC 18188) were selected and four subcultures were made from each progeny. The subcultures of F_1 and F_2 were maintained for one year by four different methods: 1) the first group of each progeny was maintained in mycelial form at 25C on Sabouraud agar by transferring once a month; 2) the second group of each strain was maintained by passing through mice only; 3) the third gorup was maintained by periodically alternating from in vivo to in vitro culture; 4) the fourth group was maintained in vitro at 37C as the yeast form on Blood-glucose-cysteine agar (BGC) and transferred once a week.

At the end of one year all the strains were cultured on soil extract agar in mycelial form to compare the intensity of colony pigment. The strains were also crossed with tester strains to produce cleistothecia. Four of the ten progeny, including 2+ and 2-, were randomly selected to compare the LD_{50} of the strains maintained by the four different methods.

B) Numerous isolates of Histoplasma capsulatum from soil and human sources of various geographic origin were obtained. The presence of their sexual state was tested by culturing them in all possible pairs on Alphacel-yeast-extract agar and incubating at 25C for 2-3 weeks. All the isolates were also cultured on Blood-glucose-cysteine agar to convert them into yeast form. The ascospores produced by mating of two compatible strains were isolated by micromanipulation, and tested for their sexual pattern by culturing singly or in pairs on Alphacel-yeast-extract agar.

Major Findings:

A) Seven of the ten strains maintained in mycelial form on Sabouraud agar lost pigment and fruiting competence completely after one year. The three remaining strains retained a tinge of buff pigment and produced pseudocleistothecia with no ascospores. Nine of the ten strains maintained by passing only through mice retained the colony pigment and produced cleistothecia abundantly. The remaining one strain lost pigment as well as fruiting competence. The third group of ten strains in vivo and in vitro cultures all produced intense pigment as well as abundant cleistothecia. The fourth group maintained in vitro at 37C on BGC agar showed a slight loss in pigmentation and produced fewer cleistothecia than the second or third group of cultures. The result of LD_{50} testing among the four groups of strains showed no correlation between the virulence, fruiting competence and the intensity of colonial pigment.

B) Mated cultures of Histoplasma capsulatum produced the cleistothecia superficially resembling those of Ajellomyces dermatitidis. Extensive observations on the development of the ascocarp revealed, however, that the perfect state of H. capsulatum differs sufficiently from all previously known genera to warrant its description as a new genus, Emmonsiella, in Gymnoascacae. Randomly isolated single-ascospores produced self-sterile but cross-fertile colonies indicating that strains of H. capsulatum are heterothallic. The single ascospore cultures produced the yeast form typical of H. capsulatum in mouse tissue and on BGC agar at 37C. The cultures fell into two cross-mating classes which were disignated as + and - types.

The isolates from soil and those of human origin mated successfully indicating their genetic compatibility.

Significance to Biomedical Research and the Program of the Institute:

A) The study of Ajellomyces dermatitidis indicates that the alternation of in vivo and in vitro passage or strictly in vivo passage using mice are the most effective methods while maintaining the strains in mycelial form at 25C on conventional agar is the poorest for the preservation of sexual vigor and pigmentation of each strain. The study also indicates that fruiting competence is directly related to the formation of buffy pigment in each strain, but not related to virulence.

B) The discovery of sexual state of Histoplasma capsulatum gave the clue to the proper taxonomic position of the fungus. It is now clear that the H. capsulatum is closer to Blastomyces dermatitidis phylogenetically than any other taxa. The discovery of heterothallism opens the road for genetic studies of the fungus.

Proposed Course of the Project:

The project will be pursued to investigate 1) the relationship between the geographic distribution of + and - type of H. capsulatum in nature and the frequency of the two types among clinical cases of human histoplasmosis and 2) the genetic control of mating system, dimorphism, colonial type and virulence of H. capsulatum.

Honors and Awards:

The author's discovery on the sexual state of H. capsulatum was selected by both Internal Medicine Digest and the American Society of Microbiology Annual Meeting Publicity Committee for news reports.

Publications:

Kwon-Chung, K. J.: Genetic analysis on the incompatibility system of Ajellomyces dermatitidis. Sabouraudia 9: 231-238, 1971.

Kwon-Chung, K. J.: Genetic study on the incompatibility of Arthroderma simmii. Sabouraudia 10: 74-48, 1972.

Kwon-Chung, K. J.: Sexual state of Histoplasma capsulatum. Science 175: 326, 1971.

Kwon-Chung, K. J.: Emmonsiella capsulata: the sexual state of Histoplasma capsulatum. Mycologia 64 (in press).

Serial No. NIAID 78-A
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Experimental Therapy of Mycoses

Previous Serial Number: Same

Principal Investigator: George W. Lones

Other Investigators: Terry J. Popkin and Benjamin Prescott

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	0/12	-	-	0/12
Professional	-	-	-	-
Other	-	-	-	-

Project Description: See 1971 Annual Report; Project terminated.

Publications:

Lones, G. W., and Popkin, T. J.: Effect of Saramycetin on the fine structure of Histoplasma capsulatum. Mycopathologia et Mycologia Applicata 46: 103-109, 1972.

Serial No. NIAID-78-B
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Physiology of *Coccidioides immitis*

Previous Serial Number: Same

Principal Investigator: George W. Lones

Other Investigators: None

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	0/12	-	-	0/12
Professional	-	-	-	-
Other	-	-	-	-

Project Description: See 1971 Annual Report; Project terminated.

Publications:

Lones, G. W., Peacock, C. F., and McNey, F. A.: Factors affecting the reversion of *Coccidioides immitis* Sperules to Mycelima. *Sabouraudia* 9: 287-296, 1971.

Serial No. NIAID 79-B-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host Response to Pathogenic Fungi in Neoplastic Disease and Immunosuppression

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: Michael Worthington (L.V.D.) Serial No. NIAID 71-H

Cooperating Units: Laboratory of Viral Diseases; and Baltimore Cancer Research Center (N.C.I.), Baltimore, Maryland

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	9/12	-	-	9/12
Professional	3/12	-	-	3/12
Other	6/12	-	-	6/12

Project Description:

Objectives:

A. to study the antibody response to fungal infection in leukemic patients, and its use in laboratory diagnosis.

B. to determine the effect of immunosuppressive or cancer chemotherapeutic drugs on experimental fungal infection in mice.

Methods Employed:

A. Serum samples are tested for antibodies to antigens of Candida albicans antigenic group A. Heat-killed whole yeast cells are used as antigen for agglutination, a soluble extract (s) from sonically disrupted viable yeast cells, and mannan (M) from yeast cells are used as antigens in immunodiffusion. For agglutination, a four-fold rise in titer is interpreted as a positive response.

The observation of a precipitin band with either soluble antigen is interpreted as a positive reaction.

B. Polyinosinic: Polycytidylic acid (In:Cn), an interferon stimulator, was tested for its effect on experimental candidiasis, coccidoiodomycosis, and cryptococcosis in mice. For each infection three groups of mice were given different courses of treatment. One group was injected with In:Cn 100 mg/injection for each animal 1 day before challenge and then every second day for a total of seven injections. The second group was given one injection only one day before challenge. The third group was injected with the In:Cn vehicle, phosphate buffered saline. Deaths were recorded as they occurred over a four week period of observation. Kidney levels of the yeast in mice infected with C. albicans were determined by quantitative plating at three and five days after challenge.

Major Findings:

A. Eighty-four serum samples from forty-three patients were tested with the Candida antigens. Positive reactions were obtained with sera from eleven patients. These positive results have been used as an index for the diagnosis of candidiasis in leukemic patients at Baltimore Cancer Research Center (N.C.I.).

B. The injection of In:Cn before and after challenge of mice with either Candida albicans or Coccidioides immitis resulted in more rapid death and a higher rate of mortality than in untreated control animals. The kidney levels of C. albicans were greater in the treated than in the untreated groups. In murine cryptococcosis, In:Cn does not appear to affect the progression of the disease.

Significance to Biomedical Research and the Program of the Institute:

A. Serology can be used to augment and enhance the chances for a laboratory diagnosis of candidiasis in immunologically compromised patients. This aids the physician in the treatment and control of the disease.

B. Poly I;C, a polynucleotide known to stimulate antiviral substances and to provide some anti-tumor activity, increases susceptibility to some experimental fungal infections. This enhancement of susceptibility must be considered in its chemotherapeutic role.

Proposed Course of the Project:

To continue to collect more information concerning the interpretation and limitations of the tests and to exchange reagents with other laboratories doing this type of testing in order to achieve standardization of the methods. To study in animal models the effect of cytotoxic drugs on experimental fungal infections.

Honors and Awards:

Chairman, Standards and Examining Committee for Certification in Medical Mycology, American Board of Medical Microbiology.

Publications:

Preisler, H. D., Hasenclever, H. F., and Henderson, C. S.: Anti-Candida antibodies in patients with acute leukemia. Amer. J. Med. 51: 352-361, 1971.

Worthington, Michael, and Hasenclever, H. F.: Effect of an Interferon Stimulator, Polysinosimic: Polycytidylic acid, on experimental fungus infections. Infection and Immunity 5: 199-202, 1972.

Serial No. NIAID 79-C-1
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ecologic and Aerobiologic Studies of Histoplasma capsulatum

Previous Serial Number: Same, +77-1-C

Principal Investigator: H. F. Hasenclever

Other Investigators: K. J. Kwon-Chung

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	36/12	-	-	36/12
Professional	4/12	-	-	4/12
Other	32/12	-	-	32/12

Project Description:

Objectives:

To study the factors that contribute to the presence or absence of Histoplasma capsulatum in soil.

Methods Employed:

A. The natural sites, under study, where H. capsulatum is known to have been present for years, are at Clarksburg, Maryland and at 7th Street and Pennsylvania Avenue, Washington, D. C. Soil samples are collected at intervals and the absence or presence of the fungus is ascertained by mouse inoculation.

B. Studies of the viability of H. capsulatum in autoclaved and raw soil continue for the third year. The relationships concerning the survival of H. capsulatum, the changes in population of bacteria and streptomycetes, and the changes of pH in soil infusion with or without bat guano have been investigated.

Soil samples, collected from eight different sites at Clarksburg, Maryland and processed as previously described, were tested for the presence of the fungus by the mouse passage technique every six months.

C. Investigations into the effect of an artificially constituted soil microflora upon the growth or survival of H. capsulatum under laboratory conditions are being done. Soil infusions are prepared by autoclaving seven kilograms of soil in seven liters of water for a total of 15 hours. The infusions were inoculated with various levels of spore suspensions made of 10 streptomycetes and cell suspensions of 10 bacterial species isolated from a soil that was negative for H. capsulatum. Conidia of H. capsulatum were added to each culture flask and incubated on a rotary shaker at room temperature. Samples were removed periodically, the pH determined, injected into mice, and plated on several media selective for the isolation of the different groups of soil microorganisms. Control flasks were made by adding H. capsulatum to sterile soil infusion with or without bat guano.

Major Findings:

A. H. capsulatum was isolated from 7th Street and Pennsylvania Avenue, a former starling roost, and from two of 80 soil samples collected. One isolation was made in the spring and one in the fall, but at different locations within the site. At Clarksburg, a former bat roost, 20 of 137 samples were positive for the fungus; but at a specific location within the site, 13 of 25 were positive. The addition of bat guano to a selected location and under field conditions does not appear to influence the spread of the fungus in soil.

B. H. capsulatum has survived for two and one half years in autoclaved soil. In raw soil, however, the fungus survived for this period only when the original soil was positive. When negative raw soils were inoculated with H. capsulatum, the fungus had disappeared from most flasks after 6-18 months. The raw soils originally positive contained markedly fewer streptomycetes than bacteria whereas most raw soils originally negative contained more streptomycetes than bacteria. Two of six soils originally negative for H. capsulatum contained fewer streptomycetes than bacteria and in these two raw soils the fungus has survived beyond two and one-half years.

C. H. capsulatum grew well and survived in soil infusion as long as the pH remained near neutral or moderately alkaline. The fungus did not survive for more than six weeks at pH 5 even if the nutritional conditions favoring growth were present. Although a bat guano filtrate stimulated the growth of streptomycetes, bacteria, and H. capsulatum, bacteria became the predominant microorganisms within two weeks. In a three way combination of bacteria, streptomycetes, and H. capsulatum in soil infusion cultures, the pH quickly became neutral or alkaline, and the fungus grew and survived for eight months, when guano was added. In a similar microbial combination without guano, H. capsulatum survived only when the bacterial population esceeded greatly that of the streptomycetes. In two-way combinations with bacteria H. capsulatum survived well without bat guano but with streptomycetes the fungus did not live for more than six weeks. Since those cultures with the highest population of bacteria yielded more H. capsulatum there seems to be no direct competition between the fungus and bacteria for nutrients.

Significance to Biomedical Research and the Program of the Institute:

H. capsulatum is known to have a sporadic distribution in nature but when found is often associated with bird or bat roosts. This project is providing basic informatin about the microbial relationships within these habitats. As more data are obtained they may permit the rational development of a biological control for this fungus in nature.

Proposed Course of Project:

This project is to be continued with further investigation into conditions that may effect the existence of H. capsulatum in nature and its relationships with soil microorganisms under laboratory conditions.

Honors and Awards:

Aerobiology Advisory Committee, United States National Committee, International Biological Program

Publications:

Hasenclever, H. F.: Histoplasmosis in Bats. Health Laboratory Science (in press).

Serial No. NIAID 79-D
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunochemical Studies on Polysaccharides from Fungi

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: William O. Mitchell

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	26/12	-	-	26/12
Professional	15/12	-	-	15/12
Other	11/12	-	-	11/12

Project Description:

Objectives:

To study the molecular structure, serologic properties, and the haptenic groups of polysaccharide or other antigens isolated from yeasts and fungi.

Methods Employed:

The serologic reactive substances are extracted from fungal cells or isolated from the culture media in which the fungi were grown. Antigenic similarity or diversity are shown by agglutination, immunodiffusion, or quantitative precipitation. The monosaccharide content of polysaccharides is identified by paper chromatography after acidic hydrolysis of the parent substances. Antisera are prepared by the injection of rabbits with fungal cells.

Major Findings:

Antigenic groups have been found in Saccharomyces telluris. Among strains of S. telluris and its asporogenous (imperfect sexual) form, Torluopsis bovina

(obtained from the Centraalbureau voor Schimmelcultures, Delft, the Netherlands) and strains of S. telluris isolated from avian sources, three serotypes have been identified. The antigenic differences can be shown by agglutination, using reciprocally cross absorbed antisera, and by extracted polysaccharides in immunodiffusion. These polysaccharides are composed of mannose only. The strains of S. telluris isolated from birds can be placed in two of the serotypes. Since the asporogenous strains of T. bovina studied were found to belong to different serotypes, sexuality does not appear to influence their antigenic composition. A system for the antigenic classification of the serotypes has not yet been devised. Preliminary results indicate that the mannans isolated from S. telluris and T. bovina are structurally related to those isolated from pathogenic species of Candida.

Significance to Biomedical Research and the Program of the Institute:

These results show that antigenic groups or serotypes have been identified in another species of yeast, and that mannans on the surface of the yeast cell determine its serologic character. More knowledge of these antigenically active polysaccharides will elucidate some of the problems and aid in the interpretation of serologic reactions used in the diagnosis of fungal diseases. These studies are defining the antigens that provoke the antibody responses in some fungal infections.

Proposed Course of Project:

To be continued studying the structure of the mannans as defined by cross serological reactions with antisera to mannans of known structure from Candida albicans, C. tropicalis, C. stellatoides, and C. parapsilosis.

Honors and Awards:

General Aspects of Medical Mycology Seminar, Department of Botany, University of Maryland, March 1, 1972.

Publications:

None.

Serial No. NIAID 79-E
1. Microbiology
2. Medical Mycology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Yeast Flora of Birds

Previous Serial Number: Same

Principal Investigator: H. F. Hasenclever

Other Investigators: None

Cooperating Units: Patuxent Wildlife Research Center, Dr. Richard Kocan

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	9/12	-	-	9/12
Professional	5/12	-	-	5/12
Other	4/12	-	-	4/12

Project Description:

Objectives:

To study the yeast flora of columbiform birds and to compare those that are isolated with similar species that are known to inhabit and cause disease in man.

Methods Employed:

The throats of live, or the throats and crops of recently sacrificed, birds are swabbed and the swabs are used to inoculate glucose-neopeptone agar plates. The plates are incubated at 37C and after 24-48 hours all the different colonial types of yeasts are picked and subcultured on agar slants. The yeasts are then identified according to the morphological and physiological characteristics given in Lodder, J. (Ed.) The Yeasts - a taxonomic study (2nd edition), North Amsterdam Publishing Company, 1970. Antigenic comparisons are made using agglutinations and precipitain reactions with strains from human sources.

Major Findings:

From 37 common pigeons, Columba livia, 42 isolations of yeasts were made. Twenty were Candida albicans, 15 Saccharomyces telluris, 2 Kloeckers apiculata, 2 C. guilliermondis, 1 C. parapsilosis, 1 Torulopsis glabrata, and 1 Hansenula anomala. Four ringed turtle doves, Streptopelia risoria, were cultured. C. albicans was cultured from 3 and C. guilliermondis from the fourth. C. guilliermondis was isolated from 1 moustache quail dove, Geotrygon mystacea. Of the 23 strains of C. albicans isolated from all birds cultured 18 belonged to antigenic group A, and 5 to group B. All 15 strains of S. telluris isolated from common pigeons belonged to the same serotype as those isolated from this species of bird and included in last years report.

Significance to Biomedical Research and the Program of the Institute:

These results expand our knowledge concerning the ubiquity of some of the yeasts that are pathogenic to man and animals and their existence in this family of birds. The common pigeon is a constant associate of man and may represent a source of contagion for these yeasts. The ratio of antigenic groups of C. albicans isolated from pigeons is very similar to the ratio of the groups isolated from man. The significance, if any, of the isolation of a single serotype of S. telluris from pigeons remains to be determined.

Proposed Course of the Project:

To be continued to determine if the number and species of yeasts isolated from Columba livia has a seasonal variation. Studies for the presence of antibodies in sera from the birds cultured against yeasts that are isolated will be done.

Honors and Awards:

None

Publications:

Hasenclever, H. F.: The consistent formation of Chlamydospores by Candida tropicalis. Sabouraudia 9: 164-166, 1971.

Serial No. NIAID-207
1. Microbiology
2. Mycoplasma
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Basic Studies on Mycoplasma

Previous Serial Number: Same

Principal Investigator: Joseph G. Tully, Ph.D.

Other Investigators: None

Cooperating Units: Laboratory of Bacterial Products, DBS (DBS-6959)
Laboratory of Parasitic Diseases, NIAID (NIAID-123B)

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	49/12	-	-	49/12
Professional	13/12	-	-	13/12
Other	36/12	-	-	36/12

Project Description:

Objectives:

The objectives of these studies cover both basic and applied aspects of Mycoplasma including their nutrition and metabolism, mechanisms of virulence, immunological interrelationships among human and animal strains, and their role in human infection and certain other mixed bacterial, viral and protozoan diseases of man and animals.

Methods Employed:

Recovery of mycoplasmas from various hosts was accomplished by standard cultivation techniques and all agents were serologically typed by fluorescent antibody or metabolic-inhibition procedures. Characterization of sterol-nonrequiring mycoplasmas was made on the basis of biochemical and serological properties and on cell protein patterns obtained by gel electrophoresis. Animal mycoplasmas were compared in a variety of biological and serological procedures to other prototype human and animal Mycoplasma strains in the

current culture collection. The gel electrophoretic pattern of Mycoplasma cell proteins was also used to confirm the serological relationships among various strains.

Major Findings:

A. Our earlier studies on sterol-nonrequiring mycoplasmas (see previous annual reports) provided the basis for a new taxonomic grouping for these organisms (Acholeplasma) and a new species in this taxon was described. Current studies have confirmed our earlier impression that the acholeplasmas represent a large group of distinct organisms that differ fundamentally from other Mycoplasma species. Approximately 15 new sterol-nonrequiring strains from a variety of hosts have been characterized in an endeavor to establish those properties that are useful in distinguishing these organisms. These strains appear to fall into five groups which can be separated by biological and serological properties. The biological properties most useful in classifying these strains include carbohydrate fermentation patterns, carotenoid production, and fermentation of aesculin. A variety of serological tests, including growth-inhibition tests have been used to compare serological relationships to establish acholeplasmas. Electrophoretic analyses with acrylamide gels of the cell protein patterns have also proved useful in grouping the new strains. These comparisons have also been of value in establishing the host origin of A. axanthum, the new species described in an earlier report. The initial isolates of this species were recovered from cell cultures and the animal origin of this particular Acholeplasma was not known. A strain recovered from the bovine nasal cavity by other investigators was found in these studies to be related to the type species of A. axanthum. Two of the new groups described here are of interest since they do not appear to be related to established Acholeplasma species. One of the groups, which consists of two related strains isolated from cattle, possesses sufficient distinctions to indicate it represents a new species. The other group consists of five serologically distinct strains but it is uncertain at this point whether they all represent new species. The widespread host distribution of these acholeplasmas and their frequent association with animal diseases (conjunctivitis, pneumonia) suggest that they may be useful in studying the pathogenicity of the sterol-nonrequiring mycoplasmas.

B. Following the reports of Gourlay and Maniloff that lysogenic viruses are present in some acholeplasmas, we examined a group of acholeplasmas and mycoplasmas in our collection for the presence of viruses. The three control virus preparations employed had a titer of 10^6 to 10^8 plaque-forming-units/ml of A. laidlawii. We were not able to demonstrate the presence of lytic viruses in the six acholeplasmas or seven mycoplasmas tested. Although further work is required to exclude the presence of viruses in these mycoplasmas, the serological heterogeneity exhibited by each of the selected strains in comparison with its established type strain, suggests that transduction of antigens from one mycoplasma serotype to another is a distinct possibility.

C. Continued study on the role of mycoplasmas in a paralytic syndrome in rats with an experimental Plasmodium berghei infection has provided new and substantial evidence that M. neurolyticum is involved in the neurological disease. Although it was never possible to show a specific antibody response

to M. neurolyticum in rats during early stages of the infection and at the time paralysis became apparent, it became clear that this short period did not provide an optimal time for adequate antibody synthesis. Since few rats survive the mixed infection after appearance of paralysis, a large number of animals were challenged with the paralytic KBG-173 strain of P. berghei. Survivors were divided into those showing transient paralysis and recovery and those without evidence of paralysis. All animals were bled at the end of two weeks. Antibody studies carried out with the metabolic-inhibition (MI) technique showed that those with transient paralysis had antibody titers to M. neurolyticum of 1:80 to 1:5120 (geometric mean 1:525). Those rats surviving challenge without evidence of paralysis had serum antibody levels of 1:10 to 1:1280 (mean 1:160). Normal rats had MI titers less than 1:10. This antibody response appeared to be specific for M. neurolyticum since we could not show the presence of antibody to M. pulmonis or M. arthritidis in these sera. Active immunization of rats with M. neurolyticum cells and toxin, as well as passive tests with rabbit antiserum prepared against mycoplasma cells and toxin, were never completely successful in blocking the paralytic P. berghei infection. A 50-60% reduction in the number of animals exhibiting paralysis was consistently observed. Additional studies were carried out on the role of the spleen in the paralytic syndrome. The major findings of this study indicated that serial passage of the KBG-173 mixed infection in splenectomized rats reduced, and eventually blocked, the incidence of paralysis in this host. Although the rate of paralysis declined during early passage in rats without a spleen, there was some indication that the agent responsible for paralysis increases in numbers since passage of blood into intact rats was followed by a high incidence of neurological disease. Removal of the spleen during early stages of the mixed infection (3-5 days post-challenge) effectively blocked the occurrence of paralysis. Splenectomy performed later in the infection (5-7 days post-challenge), and before onset of paralysis, was not effective in preventing paralysis. We believe that these findings and other information indicate that the spleen provides a site for localization of the mycoplasmas and possibly represents the organ where toxin is produced. Although removal of the spleen altered the relationship between the malaria parasites and the paralytic agent, we were still not successful in recovering mycoplasmas from the blood and tissues of splenectomized rats or from intact rats receiving blood from splenectomized donors. M. neurolyticum was also inoculated into normal rats in sub-toxic doses to study tissue distribution and persistence of mycoplasmas in the absence of an associated stress. At two hours post inoculation, only spleen cultures yielded mycoplasmas. Six hours after challenge, we were able to recover mycoplasmas from the spleens of only half the animals inoculated. Rat tissues removed at 24 hours or at later times were negative for mycoplasmas. However, rats given the same M. neurolyticum challenge and then inoculated seven days later with a non-paralytic strain (NYU_2) of P. berghei came down with the typical paralytic disease. These findings support our contention that M. neurolyticum is responsible for the neurological disease and that, although these mycoplasmas have lost the ability to grow outside the host (in culture), they are still present in host tissues and are able to multiply and produce disease when the host is sufficiently stressed.

D. Additional studies have also been carried out on several groups of animal mycoplasmas to determine their relationship to mycoplasmas of human

origin. Part of this group consisted of several mycoplasmas recovered by a collaborator from two separate outbreaks of keratoconjunctivitis in sheep and goats. An analysis of these mycoplasmas indicated that a separate and new Mycoplasma serotype was involved in the outbreak. The new species was characterized by extensive serological and biochemical tests and by acrylamide gel electrophoresis. In cooperation with other investigators these mycoplasmas have been proposed as a new species (M. conjunctivae).

Significance to Bio-Medical Research and the Program of the Institute:

The unique host specificity originally described for mycoplasmas is rapidly changing as new information techniques and the basic biology of this group of microorganisms unfolds. As a consequence of this new information, and to determine adequately the role of mycoplasmas in human diseases and in diseases of undetermined etiology, the biological and serological characteristics of all human and animal mycoplasmas must be more adequately known and described. Studies carried out on the sterol-nonrequiring mycoplasmas (acholeplasmas) can provide some important insights into membrane structure and function since exogenous cholesterol is not required by these organisms for formation of new membrane. The widespread host distribution of the acholeplasmas and their frequent association with animal disease suggest that the earlier impression that they played no role in disease might need revision. What role viruses might play in altering the antigenic characteristics of acholeplasmas is still not established but their presence in wall-less organisms opens up questions about the mechanism of virus attachment to membranes and whether a virus infection may potentiate the virulence of mycoplasmas. Mixed infections involving mycoplasmas and other agents have suggested that many mycoplasmas are able to produce disease when the host becomes stressed with a concurrent infection. Much more information is needed on the contribution of each component in this interaction. The studies on mixed infections reported here would indicate that some mycoplasmas are able to reside in the host in a non-culturable form but are still able to produce disease when the host is stressed by other means. The means by which a mycoplasma adjusts to this situation to become latent and how the agent might be activated again to grow outside of the host have important implications in human disease.

Proposed Course of Project:

Further studies are in progress on human and animal mycoplasmas and their importance to man. These studies are particularly directed to three points:

1. Are some human diseases of undetermined etiology related to animal mycoplasmas?

2. Can some mycoplasmal diseases in animals provide appropriate models for the study of the role of these agents in human diseases?

3. Are there techniques useful in demonstrating latent mycoplasmas in animal hosts that can be applied to some human diseases where mycoplasmas might be involved?

Some of the critical factors in studies of this nature involve questions about what component in mycoplasmas is responsible for virulence and how this component can be maintained in artificial systems. Attempts to learn more about mycoplasmal neurotoxin and about those procedures that might reveal latent mycoplasmas in various animal hosts are in progress.

Honors and Awards:

Member, WHO/FAO Board of Animal Mycoplasma Characterization. Participate in Board meeting in London, England; January 31 - February 1, 1972. Elected Chairman of Board.

Member, International Subcommittee on Taxonomy of Mycoplasmatales.

Chairman (1971-1972), Mycoplasmology Section, American Society for Microbiology.

Project Officer, NIAID Research Contract PH 43-67-682, University of Aarhus, Denmark (completed October 1971).

Secretary (1970-1971), NIAID Assembly of Scientists.

Convened seminar on "Mycoplasma pulmonis Model Infections" and presented paper at seminar on "The Acholeplasmas" at the 22nd annual meeting of the American Society for Microbiology, Philadelphia, Pennsylvania; April 23-28, 1972.

Invited participant at a symposium on "Pathogenic Mycoplasmas" sponsored by the Ciba Foundation, London, England; January 25-27, 1972.

Publications:

Del Giudice, R. A., Carski, T. R., Barile, M. F., Lemcke, R. M. and Tully, J. G.: Proposal for classifying human strain Navel and related simian mycoplasmas as Mycoplasma primatum sp. nov. J. Bact. 108: 439-445, 1971.

Tully, J. G., Del Giudice, R. A., and Barile, M. F.: The synonomy of Mycoplasma arginini and Mycoplasma leonis. International J. Systematic Bact. 22: 47-49, 1972.

Barile, M. F., Del Giudice, R. A., and Tully, J. G.: Isolation and characterization of Mycoplasma conjunctivae spec. nov. from sheep and goats with keratoconjunctivitis. Infect. and Immunity 5: 70-76, 1972.

Tully, J. G. and Mercado, T. I.: Mixed infections: the interaction of mycoplasmas and malarial parasites. In G. Wolstenholme (ed.), Ciba Foundation Symposium on Pathogenic Mycoplasmas. Elsevier, Amsterdam, 1972 (in press).

Serial No. NIAID 152
1. Microbiology
2. Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on antibody production and mechanisms of hyerpsensitivity

Previous Serial Number: Same

Principal Investigator: Dr. Sanford H. Stone

Other Investigators: Mr. Julius H. Goode, Jr.

Cooperating Units: 1. Dr. Marius Valsamis
Dr. Cedric Raine
Division of Neuropathology
Albert Einstein College of Medicine
New York, New York

2. Dr. Raymond Steblay
New York State Kidney Research Institute
Albany, New York

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	24/12	6/12	-	30/12
Professional	24/12	-	-	24/12
Other	-	6/12	-	6/12

Project Description:

Project Subtitle I: Microbial and tissue antigens in pathogenesis of "allergic" kidney diseases (in collaboration with Cooperating Unit #2)

Objectives:

To elucidate the mechanisms underlying susceptibility to "autoimmune" renal tubule disease and "autoimmune" glomerulonephritis in guinea pigs.

Methods Employed:

Random bred and inbred histocompatible guinea pigs were sensitized with rabbit renal tubule suspensions in various adjuvant preparations. Urines were

monitored for blood, protein, sugar, and pH and kidneys were taken at appropr times after sensitization. Serum was tested by fluorescent antibody techniqu for its ability to fix to kidney tissue, and frozen kidney slices were tested for antibody in the tubules. Histologies were performed on preserved tissue

Major Findings:

Autoimmune renal tubule disease was induced by rabbit renal tubule antig incorporated in complete Freunds adjuvant, incomplete Fruends adjuvant, and aluminum hydroxide (alum) adjuvant. Strain 13, but not Strain 2, guinea pig were susceptible and older (1 year +) guinea pigs were resistant. Attempts at transfer of disease with living lymph node cells at five days and seventeer days after sensitization of donors did not succeed, but several recipients of ten day transfers showed glucose in their urines.

Significance to Bio-Medical Research and the Program of the Institute:

"Allergic" kidney disease following infectious diseases is a sufficiently common occurrence to warrant some attention as to genetics and mechanisms. The role of bacteria as the source of inducing antigens in complexes fixing to kidney tissues, or as adjuvants in autoimmune disease of the various kidney tissue components still awaits some clarification. Renal tubule disease of guinea pigs, not requiring mycobacteria in the Freunds adjuvant, may be the first good guinea pig model of an autoimmune disease induced by circulating antibodies.

Proposed Course of Project:

Transfers of living lymphoid cells and serum and combinations of fractio of these will be undertaken to delineate the mechanisms of the renal tubule disease.

Project Subtitle II: Genetics and pathogenesis of acute and chronic autoimmun encephalomyelitis.

Objectives:

To elucidate the mechanisms underlying resistance to autoimmune encephalo myelitis (AE) linked to age, sex, or strain differences in guinea pigs, in search for information on the etiology of the disease or of demyelinating diseases in general. (In collaboration with Cooperating Unit #1)

Methods Employed:

Young and mature, male and female, Strain 2 and Strain 13 guinea pigs were sensitized with homologous spinal cord incorporated into Freund's complete adjuvant (FCA) containing killed Mycobacterium tuberculosis. The effects of subsequent active or passive immunization with the same antigen were observed. Tissues from guinea pigs with acute, sub-acute, and chronic autoimmune encephalomyelitis were taken for detailed comparison of these conditions.

Major Findings:

Strain 13 guinea pigs injected with spinal cord in FCA at an early age whose particular susceptibility would ultimately (2 months later) result in chronic AE do not show acute disease which injected later with the same materials at an age which is susceptible to acute AE. Instead, the onset of the chronic AE is delayed. Similarly, actively immunized Strain 13 guinea pigs in the latent period for chronic AE are protected against acute AE which could normally be induced by adoptive transfer of lymphoid cells from sensitized isologous donors.

Significance to Bio-Medical Research and the Program of the Institute:

Besides the obvious importance of knowledge on the possible interaction of acute and chronic states in demyelinating diseases which may be of "allergic" or autoimmune etiology, some basic attitudes on immunization can be re-examined. From the previous results, it has become clear that injection of antigen as an alum precipitate or in incomplete Freunds adjuvant can deviate the immune response from the path it might take after a subsequent injection of the antigen in complete adjuvant. However, there has been little concern when protocols are designed for what might happen if the injection of an antigen in CFA is succeeded by another just like it; indeed, this is a common procedure accompanied by the attitude "if one is good, two will be better."

Proposed Course of Project:

Living lymphoid cells from acute, sub-acute, and chronic AE guinea pigs will be assayed in tissue culture and results correlated with the particular pathology of the varieties of AE.

Project Subtitle III: Studies on cellular manifestations of hypersensitivity and immunity in guinea pigs; transplantation of immunologically competent tissues.

Objectives:

Correlation of the developments in lymphoid tissues draining the sites of injection of antigens with the capacity of the lymph node cells to transfer either hypersensitivity or antibody producing capacity.

Methods Employed:

Immune deviation (suppression of susceptibility to delayed hypersensitivity) was established by injection of alum precipated antigens in guinea pigs. In appropriate passive versions of the model, guinea pigs were sensitized with various antigens incorporated into complete or incomplete Freund's adjuvant or in alum adjuvants. They are used as donors of recipients of lymphoid cells sensitized to the same antigen which were originally incorporated in the same or different adjuvant. In this way, the role of the adjuvant can be isolated and characterized.

Major Findings:

Immune deviation, once obtained by prior injection of bovine gamma globulin precipitated by alum, is not overcome by the subsequent injection of bovine gamma globulin with massive quantities of mycobacterial adjuvant. Guinea pigs receiving as much as 5 mg of killed M. tuberculosis still showed strikingly diminished delayed hypersensitivity to BGG. Animals thus deviated also were resistant to development of Jones-Mote reactivity(Cutaneous Basophil Hypersensitivity). If BGG in alum as initial stimulus was followed by BGG in incomplete Freunds adjuvant, Jones-Mote reactivity was diminished in a significant percentage of skin-tested random-bred guinea pigs.

Significance to Bio-Medical Research and the Program of the Institute:

The types of cells required for transfer of adoptive immunity are of interest for the reconstruction of the immune response at the cellular level. Which cells are susceptible to immune deviation and the mechanism of immune deviation may be queried by this approach. The adoptive transfer is a powerful tool for study of the cell-cell interactions recently recognized of great importance in immune responses. The importance of the initial adjuvant stimulation observed in deviation studies is of obvious significance to those in search of more efficient vaccines.

Proposed Course of Project:

Transfers involving cell suspensions of known composition and of slices of lymphoid tissue from sensitized deviated donors will be made into normal or deviated recipients to assess role of cell-cell association or of certain tissue architecture in normal or deviated immune responses.

Honors and Awards:

Associate Editor, Journal of Immunology

Professorial Lecturer, Howard University, Washington, D. C.

Invited Lecture: "Age and Strain-dependent Autoimmune Disease in Histocompatible Inbred Guinea Pigs," Albert Einstein College of Medicine, New York, New York, January 7, 1971.

Publications:

Davey, M. Jean, Asherson, G. L., and Stone, S. H.: Selective and specific inhibition of 24 hour skin reactions in the guinea pig. III. Depression of cytophilic and haemolytic antibodies by pretreatment with antigen and the effect of irradiation. Immunology 20: 513-522, 1971.

LABORATORY OF BIOLOGY OF VIRUSES

1972 Annual Report

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Annual Report of the Laboratory of Biology of Viruses
National Institute of Allergy and Infectious Diseases, NIH
July 1, 1971 through June 30, 1972

The general area of research of this laboratory continues to be molecular virology. Members of the Laboratory of Biology of Viruses have used knowledge of molecular biology in order to gain an understanding as to how human and animal viruses affect cells and how they replicate. Studies on the mechanism of transformation of cells by oncogenic viruses continue as a major area of investigation. This past year has been successful and during this period many notable findings have been made.

Metaphase Cells Show Unique Changes in their Chromatin Composition.

The non-histone chromosomal proteins are found to contain enzymes responsible for histone protein modification reactions. One of these, a lysine-rich (F1) histone phosphokinase was found to be unique in metaphase vs. interphase chromosomes. Its activity as monitored both in vivo, as the existence of a highly phosphorylated F1-histone and in vitro by its ability to transfer γ-^{32}P for ATP to F1-histone is greatly augmented (6-10 fold) in metaphase chromatin. This activation is independent of cyclic nucleotide stimulation and is transient in its appearance in vinblastine or Colcemid arrested mitotic cells. Although there is increased phosphorylation of histone F1 in metaphase chromosomes, no concomitant difference in DNA-conformation has yet been found by circular dichroism. Similarly, melting profiles, which indicate the distribution and interaction of proteins with DNA, showed that the basic structure of metaphase and interphase chromosomes is the same. (Lake, Shih, Salzman)

There is a Specific Initiation Site for the Replication of SV40 DNA.

Newly synthesized DNA strands have been obtained from replicating molecules by treatment with alkali. These DNA strands have then been sized by velocity gradient centrifugation and used in renaturation studies. It has been observed that the rate of renaturation increases as the size of newly synthesized DNA decreases. These results are those expected if the genetic complexity of the DNA increases as DNA replication proceeds. The relationship has been observed for molecules that are 10 to 90% replicated. These data establish that DNA replication is not initiated randomly, but rather is initiated at a specific site. (Salzman, Sebring)

The Mechanism of Replicating SV40 DNA has been Further Clarified.

Studies have been carried out on (1) the role of the interrupted, circular form of SV40 DNA (DNA II) in replication, (2) determination of a specific origin and termination for replication using bacterial restriction nucleases, and (3) the mechanism for SV40 DNA chain growth. Pulse-labeled DNA II was isolated and shown to contain the newly synthesized DNA in the interrupted strand. The relative pool size for this intermediate in replication was found to be quite small in relation to the earlier intermediates and indicated that DNA II was not a rate limiting intermediate. In order to define the origin or initiation site for DNA replication, young and mature replicating molecules have been isolated and cleaved with the restriction

endonuclease of E. coli B. The results demonstrated cleavage occurring only in the unreplicated region of the molecules and an unusual dissociation reaction taking place after cleavage in which the newly synthesized strands separated from the template strands. The results indicate that the site of cleavage may be at either the initiation site or a replication fork and that intact template strands are required at this site in order to stabilize the secondary structure of the replicative intermediate. In studies on the mechanism of DNA chain growth a 4 S DNA chain intermediate has been identified and the results suggest that a discontinuous mechanism is involved in SV40 DNA chain growth. (Fareed, Salzman)

SV40 DNA Fragments Are Being Used to Map Messenger RNA Molecules.

The restriction endonuclease from H. influenzae produces a set of unique fragments of SV40 DNA which sediment between 9s and 4s in neutral sucrose gradients. About 9 of the 11 fragments produced are resolved by electrophoresis in sodium dodecyl sulfate (SDS) buffer in 2.1% polyacrylamide-0.7% agarose gels. DNA fragments have been used as templates for RNA polymerase. While only very small amounts of RNA are produced in the high salt buffer which is generally used to promote selective transcription of intact SV40 DNA, increased amounts of synthetic RNA which hybridize to denatured SV40 DNA, are produced in a low salt buffer. All of the synthetic RNA is smaller than tRNA and 20% or more is symmetric. Competition-hybridization experiments show that in vivo late RNA from SV40 infected Vero cells competes with the synthetic RNA made from the 4 largest DNA pieces. (DeFilippes)

SV40 Integration in Mammalian Cells in Abortive Transformation and in Transformed Cell Lines.

A series of "abortive" transformants were tested for the presence of SV40 DNA. These are mouse cell lines which initially exhibited evidence of "early viral functions" such as T antigen synthesis and growth potential in depleted medium, but subsequently regained the growth and immunologic properties of normal cells. Experiments carried out with DNA prepared from an abortive transformant suggest that the SV40 DNA in the abortively transformed line is linked to mammalian DNA by alkali stable bonds. An important question facing tumor virologists is whether integration of oncogenic viral DNA occurs at specific or random sites in the chromosome(s) of mammalian cells. To answer this question the host sequences adjacent to the integrated viral DNA have been examined. The results of this experiment strongly suggest: (1) Although the host sequence adjacent to the integrated viral DNA contain predominantly non-repetitious DNA in each line examined, the site of integration is different in each of the transformed cells examined. (2) In lines containing more than one copy of SV40 DNA, the integrated viral DNA's are not integrated in tandem. (3) The SV40 genome is not integrated as small DNA fragments. (Gelb, Martin)

SV40 Transcription Has Been Studied With Separated SV40 DNA Strands.

The strands of SV40 DNA were separated by incubating P^{32}-labeled SV40 DNA

with an excess of SV40 cRNA made in vitro with E. coli RNA polymerase. The separated strands did not self anneal to any appreciable extent. The in vitro RNA reacted exclusively and completely with one DNA strand (minus strand). The viral specific RNA present in African green monkey cells late in productive infection reacted with 60-65% of the plus strand and 35-40% of the minus strand which is equivalent to the theoretical maximal gene activity. The RNA present in infected monkey cells prior to viral DNA synthesis (designated "early" RNA) reacts exclusively with the minus strand of SV40 DNA. (Khoury, Martin)

Electrophoretic Differences in the Capsid Proteins of SV40 Plaque Size Mutants.

The major structural component of SV40 is the capsid protein comprising over 75 per cent of the total virion. Comparison of the capsid proteins of the large and small plaque SV40 mutants by the isoelectric focusing technique revealed a distinct difference in their isoelectric points. By this technique, it has also been possible to distinguish the capsid proteins of a minute-plaque mutant from the large-plaque type SV40 mutant. In contrast, the capsid proteins of the small and minute plaque SV40 strains appear to be generally similar, and thus far cannot be distinguished by their isoelectric points. Differences in the tryptic digests of the capsid proteins from the small and large plaque type SV40 mutants offer an explanation for the difference in the electrophoretic pattern of the capsid proteins. (Barban)

Mechanisms Involved in Kilham Rat Virus Synthesis have been Further Defined.

KRV has entered rat nephroma cells within 1 hr after infection. New infectious virions can be detected at 12 hours and the maximal yield of virus is attained by 23 hours after infection. During this 23 hour period of virus growth, the rate of protein synthesis remains 75 to 100% of that in the uninfected cell while RNA synthesis is maintained at 100 to 150% that found in the control cells. The addition of the inhibitor of DNA synthesis, FUDR, up to 8 hours after infection completely suppresses virus production. After 8 hours, viral DNA production has started and FUDR inhibition progressively decreases until by 23 hours the addition of the inhibitor no longer causes a reduced virus yield. Viral DNA synthesis precedes by 4 hours the production of infectious virions. Concerning the mechanism of DNA synthesis, within 1 hour after infection, some of the single stranded linear virion DNA is converted into a double stranded form. After several hours both single and double stranded DNA with the sedimentation characteristics of viral DNA are produced and continue to be produced until the time of cell lysis. (Lois Salzman)

Vaccinia Virus Contains a Unique Glycoprotein Component.

Soon after vaccinia infection, synthesis of host glycoproteins is inhibited and several new glycoproteins are made. One of the virus induced glycoproteins is a component of the viral envelope while the others may be associated with cell membranes. The envelope glycoprotein contains glucosamine and has a molecular weight of 40,000. Unlike glycoproteins found in other

viruses, the vaccinia envelope glycoprotein lacks fucose, mannose, galactose and sialic acid. (Garon, Moss)

A New Enzyme which Phosphorylates Specific Viral Proteins was Found in Vaccinia Virus Particles.

The activity of the protein kinase was dependent on disruption of the viral envelope with a non-ionic detergent. Serine and threonine residues of a specific 15,000 molecular weight polypeptides were phosphorylated. Both the enzyme and the phosphate acceptor proteins were localized within the virus core. This enzyme may be important for the assembly and maturation of vaccinia virus or may regulate other core-associated enzymes during early stages of infection. (Paoletti, Moss)

The Proteolytic Enzyme, Trypsin, has been Used as a Probe of the Surface Structure of Vaccinia Virus.

Incubation of purified vaccinia virus with trypsin resulted in the nearly complete removal of several polypeptides without significant loss of DNA or lipid. The treated virus preparations retained full infectivity but appeared altered on electron microscopic examination. (Garon, Moss)

A New Method for the Separation of Protein Subunits was Discovered.

This consists of adsorbing polypeptide-sodium dodecyl sulfate complexes on to hydroxylapatite columns and eluting with a gradient of sodium phosphate and detergent. A unique feature of this method is that the polypeptide-SDS complexes do not elute in order of molecular weight. The technique has been applied to the preparative and analytical separation of vaccinia virus structural polypeptides. (Moss)

Studies with Rifamycin Derivatives have Defined the Portions of the Molecule Necessary for Anti-poxviral Activity.

Measurements of the specific anti-viral activities of a series of rifamycin derivatives indicated that only the macrocyclic ring portion of rifampicin is essential for anti-viral activity. Nevertheless, the anti-viral activity can be greatly modulated by small changes at the 4 position of the piperazinyl-iminomethyl ring. The compound 3-(4-amino-piperazinyliminomethyl) rifamycin SV was found to be effective against vaccinia virus in tissue culture at even lower concentrations than rifampicin. (Moss, LBV; Grimley, NCI)

Further Understanding of Structure of Human Adenovirus-associated Viruses (AAV) and their Mode of Replication has been Gained.

Viral RNA is synthesized from only one of the two DNA strand species during infection. The two complementary strand species of 5-bromodeoxyuridine-substituted, adenovirus-associated virus type 2 DNA were preparatively separated and the base composition of the "heavy" and "light" strand species determined. It was found that the base composition of in vivo-RNA was similar to that of light strands, and this RNA apparently hybridized only with heavy strands. These observations indicate that the heavy-strand

species alone serves as the transcriptional template in vivo. This study represents the first instance in which the base composition and specificity of in vivo transcription have been determined for each of the complementary strands of an animal virus DNA. RNA synthesized in KB cells co-infected with AAV and either adenovirus or herpes simplex virus was compared. As is the case for AAV RNA synthesized with helper adenovirus the AAV RNA synthesized with herpes simplex virus as helper annealed only to the thymidine-rich (minus) AAV DNA strand. This RNA was also identical in all other properties that were compared. (Rose, Carter)

AAV Virions are Composed of Three Structural Proteins.

The structural proteins of adenovirus-associated virus types 1, 2, and 3 were analyzed by acrylamide gel electrophoresis. In each case, one major protein (C) and two minor proteins (A and B) were identified. Component C had an estimated molecular weight of 62,000 daltons, and the molecular weights of components A and B were found to be 87,000 and 73,000 daltons, respectively. The relative incorporation of ^{14}C-arginine and ^{14}C-mixed amino acids into the three polypeptides of AAV-2 was similar, indicating an absence of an arginine-rich component. In addition, AAV-2 was found to have a substantially lower arginine content than helper adenovirus. (Rose, LBV; Maizel, Albert Einstein College of Med.; Shatkin, Roche Inst.)

Physical and Biochemical Techniques are being Used to Analyze Structure of the Adenovirus Genome.

Human adenoviruses contain double-stranded, linear DNA with a molecular weight of 20-25 x 10^6 daltons. Because native DNA molecules do not form circles on annealing after treatment with E. coli exonuclease III, others have concluded that the adenovirus genome does not contain terminally repetitious nucleotide sequences. However, electron microscopic studies with DNA from several adenovirus serotypes have revealed that single-stranded, circular molecules are formed when native adenovirus DNA is denatured and then annealed. These single-stranded circles do not form if a limited number of nucleotides (3-5%) are removed from the 3' ends of the native molecules by exonuclease III digestion prior to denaturation and annealing. It is therefore concluded that adenovirus DNA does have terminally repeated sequences, but these sequences are inverted. As a result, each DNA strand contains complimentary, terminal sequences which may self-anneal to form the observed single-stranded circles. This mode of terminal repetition has not been observed previously. From these and continuing studies of AAV and adenovirus structure and synthesis, it is hoped that a greater understanding of mechanisms regulating viral replication can be gained. Knowledge of these processes at the molecular level could serve as a basis for developing biochemical controls of viral infection. (Garon, Rose)

Identification of RNA Tumor Virus Genetic Material in Animal Cells.

Both normal and transformed cells were examined for the presence of murine leukemia-sarcoma virus-specific DNA employing H^3-labeled DNA prepared in vitro with the RNA dependent DNA polymerase associated with Kirsten sarcoma virus. The in vitro labeled DNA probe represents nearly all of the genetic information encoded in the 70S RNA. In the normal and transformed

mouse lines examined approximately 11 copies of viral genetic information were found. Under the experimental conditions employed only 0.4 copies of viral DNA were found in monkey cells. Within the limits of the assay, it was not possible to detect any difference in the amount of RNA tumor virus-specific DNA in normal and transformed cells. A series of murine embryo cells from AKR, C-57, NIH Swiss and Balb mice (which form a spectrum in their ability to spontaneously release leukemia viruses) were tested for the presence of virus-specific DNA. Approximately 12-14 copies of viral genetic material was found in each line. (Martin, Gelb, LBV; Aaronson, NCI)

Serial No. NIAID-80A
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Characterization of Nucleic Acids of Transformed and Virus Infected Cells

Previous Serial No.: Same

Principal Investigators: Dr. Malcolm A. Martin
Dr. Lawrence Gelb
Dr. George Khoury

Other Investigators: Ms. Janet Byrne

Cooperating Units: Dr. Kenneth Takemoto, LBV, NIAID
Dr. Stuart Aaronson, VCB, NCI
Dr. Helene Smith, Dept. of Microbiology, University of California, Berkeley, California

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	60/12			60/12
Professional:	32/12			32/12
Other:	28/12			28/12

Project Description:

Objectives:

To elucidate the process of viral DNA transcription under conditions of productive and transforming infection.

To examine the mechanisms involved in the integration of viral genetic material in the animal cell genome.

To detect and characterize RNA tumor virus genetic material in normal and transformed cells by utilizing labeled in vitro DNA made with the virus-associated RNA dependent DNA polymerase.

Methods Employed:

SV40 Integration on Mammalian Cells. Quantitation of SV40 DNA equivalents in mammalian cells was carried out by measuring the effects of unlabeled transformed cell DNA on the reassociation of P^{32} SV40 DNA. "Integrated" viral DNA was separated from free SV40 DNA by sedimenting preparations of gently lysed mammalian DNA through a 5-40% alkaline sucrose gradient in a TI-15 zonal centrifuge rotor. The gradients generated (1400-1600 ml) afforded a convenient method to fractionate large volumes of high molecular weight mammalian DNA.

SV40 DNA Transcription. The individual strands of SV40 DNA were preparatively separated by incubating denatured, linear P^{32}-labeled SV40 DNA with a large excess of SV40 complementary RNA (cRNA) prepared *in vitro* with *E. coli* RNA polymerase. Incubation conditions were chosen to minimize the reannealing of the labeled viral DNA. The entire reaction mixture was applied to hydroxyapatite (HAP) columns and the SV40 DNA strand by differential salt elution. The proportion of each viral DNA strand transcripted *in vivo* was determined by allowing DNA to react with an excess of mammalian cellular RNA and subsequent analysis of the products formed on HAP.

Major Findings:

SV40 Integration in Mammalian Cells. In collaboration with Dr. Helene Smith, we examined a series of "abortive" transformants for the presence of SV40 DNA. These are mouse cell lines which initially exhibited evidence of "early viral functions." Such as T antigen synthesis and growth potential in depleted medium, but subsequently regained the growth and immunologic properties of normal cells. In two of three of these lines we detected between 2 and 6 copies of SV40 DNA. In order to determine whether the viral genetic material in the abortive transformants existed in the same physical state as the viral genome in SV40 induced tumor cells, we performed sedimentation analyses of high molecular weight transformed cell DNA's in a zonal rotor. When the total cellular DNA from a standard transformant (11A8) was examined, 1.96 viral DNA equivalents were detected. Approximately 2.1 copies of SV40 DNA was associated with the rapidly sedimenting high molecular weight transformed cell DNA. In a similar experiment carried out with DNA prepared from an abortive transformant that contained 5.5 equivalents, about 7.2 copies of viral DNA were detected in the high molecular weight fraction. This experiment suggested that the SV40 DNA in the abortively transformed lines was linked to mammalian DNA by alkali stable bonds. In another series of experiments to determine whether integration of viral genetic material occurred during productive infection of African green monkey cells with SV40, we detected approximately 4.2 copies of viral DNA associated with the high molecular weight animal DNA 24 hours following infection.

An important question facing tumor virologists is whether integration of oncogenic viral DNA occurs at specific or random sites in the chromosome(s) of mammalian cells. We have attempted to answer this question by examining the host sequences adjacent to the integrated viral DNA. DNA

was prepared from a series of virus-free transformed cells, mechanically sheared to fragments of 8 x 10^6 or 7 x 10^5 daltons (the molecular weight of SV40 DNA is 3 x 10^6 daltons) and then fractionated into repetitious and nonrepetitious fractions. We examined both fractions of each DNA preparation (at the two different fragment sizes) for the presence of SV40 DNA. The results of this experiment strongly suggest: 1) Although the host sequences adjacent to the integrated viral DNA contain predominantly nonrepetitious DNA in each line examined, the site of integration is different in each of the transformed cells examined. 2) In lines containing more than one copy of SV40 DNA, the integrated viral DNA's are not integrated in tandem. 3) The SV40 genome is not integrated as small DNA fragments.

SV40 DNA Transcription. The strands of SV40 DNA were separated by incubating P^{32}-labeled SV40 DNA with an excess of SV40 cRNA made in vitro with E. coli RNA polymerase. The separated strands did not self anneal to any appreciable extent. The in vitro RNA reacted exclusively and completely with one DNA strand (minus strand). The viral specific RNA present in African green monkey cells late in productive infection reacted with 60-65% of the plus strand and 35-40% of the minus strand which is equivalent to the theoretical maximal gene activity. The RNA present in infected monkey cells prior to viral DNA synthesis (designated "early" RNA) reacts exclusively with the minus strand of SV40 DNA.

Identification of RNA Tumor Virus Genetic Material in Animal Cells. We have examined both normal and transformed cells for the presence of murine leukemia-sarcoma virus-specific DNA employing H^3-labeled DNA prepared in vitro with the RNA dependent DNA polymerase associated with Kirsten sarcoma virus. Our in vitro labeled DNA probe represents nearly all of the genetic information encoded in the 70S RNA. In the normal and transformed mouse lines examined we found approximately 11 copies of viral genetic information. Under the experimental conditions employed we detected only 0.4 copies of viral DNA in monkey cells. Within the limits of the assay, we were unable to detect any difference in the amount of RNA tumor virus-specific DNA in normal and transformed cells. We also examined a series of murine embryo cells from AKR, C-57, NIH Swiss and Balb mice (which form a spectrum in their ability to spontaneously release leukemia viruses) for the presence of virus-specific DNA. Approximately 12-14 copies of viral genetic material was found in each line.

Significance to Biomedical Research and the Program of the Institute:

The ultimate goal of this project is to understand the integration of viral genetic material into the mammalian chromosome and the relationship of this process to oncogenesis. The determination of the time course of integration, the number of copies of viral DNA present in cells undergoing transforming infection and the nature of the integration site in the animal cell genome may permit one to separate the various steps involved and possibly interrupt these molecular events with appropriate chemotherapeutic agents.

We are also interested in delineating those viral genes necessary to induce and/or maintain the transformed state. Utilizing the separated strands of SV40 DNA, we will be able to characterize those viral gene products that are present in tumor cells from those required for SV40 productive infection. A more thorough understanding of the control mechanisms which modulate viral DNA transcription may enable one to regulate the type of cellular response to these agents.

Proposed Course of the Project:

Emphasis will be placed on understanding the mechanisms of integration of both RNA and DNA tumor virus genetic material into the host chromosome(s). Mammalian DNA from cells undergoing both productive and transforming infection will be examined to determine when stable integration occurs. We are also in the process of characterizing the viral-specific genetic information shared by RNA tumor viruses and normal cells. Utilizing the separated strands of SV40 DNA we are examining the pattern of viral transcription in a series of transformed cell lines derived from several different mammalian species.

Honors and Awards:

Invited lectures at:

University of California, San Francisco, California

Harvard University, Cambridge, Massachusetts

Gave a two-week course for 20 students entitled "Tumor Virus Workshop," August, 1971, Cold Spring Harbor, N. Y.

Gave a one-week course for 12 students entitled "Biosynthesis of Macromolecules," January, 1972, Caracas, Venezuela.

Publications:

Gelb, L. D., Aaronson, S. A. and Martin, M. A.: Heterogeneity of murine virus in vitro DNA. Science 172: 1353-1355, 1971.

Gelb, L. D. and Martin, M. A.: The detection and quantitation of vora; genomes within normal and transformed mammalian cells. Symp. on Embryonic and Fetal Antigens in Cancer. N. G. Anderson and J. H. Coggin, J., Eds., 71-84, 1971.

Stone, L. B., Takemoto, K. K. and Martin, M. A.: Physical and biochemical properties of progressive pneumonia and visna viruses. J. Virol. 8: 573-578, 1971.

Smith, H. S., Gelb, L. D. and Martin, M. A.: Detection and quantitation of simian virus 40 genetic material in abortively transformed BALB/3T3 clones. Proc. Natl. Acad. Sci. USA 69: 152-156, 1972.

Serial No. NIAID-80G
1. Biology of Viruses
2. Macromolecular Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Regulation of Viral Protein Synthesis and Assembly of Virus Particles

Previous Serial No.: Same

Principal Investigators: Dr. Bernard Moss
Dr. Enzo Paoletti
Dr. Hortencia Rosemond
Miss Edith Rosenblum
Mr. Norman Cooper

Other Investigators: Dr. Claude Garon

Cooperating Units: Dr. Philip M. Grimley, Laboratory of Pathology, N.C.I.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	56/12		8/12	64/12
Professional:	34/12		8/12	42/12
Other:	22/12			

Project Description:

Objectives:

1. Determine regulatory mechanisms involved in synthesis of viral proteins.

2. Analyze post-translational modifications consisting of polypeptide cleavage and the addition of carbohydrate and phosphate groups.

3. Elucidate the mechanisms involved in the assembly of virus particles.

4. Define the physical and functional relationships of polypeptides comprising the virion.

5. Determine the modes of action of anti-viral drugs.

Methods Employed:

Vaccinia virus was grown in HeLa cells and purified by density gradient sedimentation. DNA, RNA, protein and carbohydrate synthesis were measured by the incorporation of radioactive precursors and the separation of macromolecules by gel electrophoresis, gel filtration, column chromatography and zonal centrifugation. Further analysis of purified components were performed by chemical and enzymatic hydrolysis and then larer chromatography and electrophoresis. The electron microscope was used as a tool to study viral morphogenesis.

Major Findings:

Vaccinia virus contains a unique glycoprotein component. Using radioactively labeled glucosamine as a specific carbohydrate precursor, a single glycoprotein component was identified. The glycoprotein has a molecular weight of 40,000 daltons and is a component of the viral envelope. Unlike glycoproteins found in other viruses, the vaccinia glycoprotein does not appear to contain fucose, mannose, galactose or sialic acid. The glycopeptide portion of the protein, after pronase digestion, was isolated by gel filtration. Synthesis of the glycopeptide does not occur in uninfected cells. The glycopeptides isolated from vaccinia virus grown in avian and mammalian cells were distinguishable.

A new enzyme which phosphorylates specific viral proteins was found in vaccinia virus particles. A protein kinase was discovered in vaccinia virus by incubating purified virus with γ-^{32}P-ATP. Enzymatic activity was dependent on pH, divalent cations, dithiothreitol and disruption of the viral envelope with a non-ionic detergent. Further analysis showed that serine and threonine residues of specific 15,000 molecular weight polypeptides were phosphorylated. Both the enzyme and the phosphate acceptor proteins were localized within the virus core. The enzyme can also phosphorylate histones when they are added to the reaction mixture. This enzyme may be important for the assembly and maturation of vaccinia virus or may regulate other core-associated enzymes during early stages of infection.

The proteolytic enzyme, trypsin, has been used as a probe of the surface structure of vaccinia virus. Incubation of purified vaccinia virus with trypsin resulted in the nearly complete removal of several polypeptides without significant loss of DNA or lipid. The treated virus preparations retained full infectivity but appeared altered on electron microscopic examination.

A new method for the separation of protein subunits was discovered. This consists of adsorbing polypeptide-sodium dodecyl sulfate complexes on to hydroxylapatite columns and eluting with a gradient of sodium phosphate and detergent. A unique feature of this method is that the polypeptide-SDS complexes do not elute in order of molecular weight. The high resolution of the method was demonstrated by separating the α and β chains

of hemoglobin. The technique has been applied to the preparative and analytical separation of vaccinia virus structural polypeptides.

Studies with rifamycin derivatives have defined the portions of the molecule necessary for anti-poxviral activity. The anti-viral action of a series of rifamycin derivatives was measured by plaque inhibition, by inhibition of wild-type versus mutant virus yields and by specific interruption of virus morphogenesis. These studies indicated that only the macrocyclic ring portion of rifampicin is essential for anti-viral activity. Nevertheless, the anti-viral activity can be greatly modulated by small changes at the 4 position of the piperazinyliminomethyl ring. The compound 3-(4-amino-piperazinyliminomethyl) rifamycin SV was found to be effective against vaccinia virus in tissue culture at even lower concentrations than rifampicin.

Significance to Bio-medical Research and the Programs of the Institute:

Poxviruses infect many vertebrate species and are human pathogens. These studies have provided fundamental information regarding the synthesis of component structures of poxviruses and their assembly into virus particles. Such information can provide insights for the development of chemotherapeutic agents and for studying related processes in normal cells and other virus-cell systems. One antibiotic studied, rifampicin, has been found to specifically interrupt vaccinia virus assembly and derivatives of this drug may prove to have therapeutic value.

Proposed Course of the Project:

Work will continue on the characterization of polypeptides induced during vaccinia virus infection. Emphasis will be placed on their biosynthesis, post-translational processing, and assembly into virions. Attempts will be made to isolate and purify the protein kinase and other enzymes localized within the virus core.

Honors and Awards:

Appointed to the Editorial Board of Journal of Virology.

Invited speaker at 1971 Gordon Research Conference on Animal Cells and Viruses.

Lecture in course on Developmental Biology in the NIH Graduate Program.

Publications:

Moss, B., Rosenblum, E. N. and Grimley, P. M.: Assembly of vaccinia virus particles from polypeptides made in the presence of rifampicin. Virology 45: 123-134, 1971.

Moss, B., Rosenblum, E. N. and Grimley, P. M.: Assembly of virus particles during mixed infection with wild-type vaccinia and a rifampicin-resistant mutant. Virology 45: 135-148, 1971.

Moss, B., Rosenblum, E. N. and Garon, C. F.: Glycoprotein synthesis in cells infected with vaccinia virus. I. Non-virion glycoproteins. Virology 46: 221-232, 1971.

Garon, C. F. and Moss, B.: Glycoprotein synthesis in cell infected with vaccinia virus II. A glycoprotein component of the virion. Virology 46: 233-246, 1971.

Grimley, P. M. and Moss, B.: Similar effects of rifampin and other rifamycin derivatives on vaccinia virus morphogenesis. J. Virology 8: 225-231, 1971.

Moss, B. and Rosenblum, E.N.: Hydroxylapatite chromatography of protein-sodium dodecyl sulfate complexes: a new method for the separation of polypeptide subunits. J. Biol. Chem. In Press.

Serial No. NIAID-80H
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Characterization and Replication of Kilham Rat Virus

Previous Serial Number: Same

Principal Investigator: Dr. Lois A. Salzman

Other Investigators: Mr. Wesley White

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	34/12			34/12
Professional:	12/12			12/12
Other:	22/12			22/12

Project Description:

Objectives:

1. To study the production of virus and the synthesis of viral DNA after infection of a rat nephroma cell line with Kilham rat virus (KRV). The virion contains a single-stranded linear DNA molecule and replicative intermediates in DNA synthesis will be looked for.

2. To determine the fate of the virion DNA after infection of a susceptible host.

3. To examine the effect of KRV infection on cellular RNA and protein synthesis.

4. To solubilize, purify and further characterize any eznymes associated with the purified virions.

Methods Employed:

The growth of Kilham rat virus (KRV) in a rat nephroma cell line was examined by infecting the cells, and at various times after infection,

examining the cells and the extracellular media for the presence of infectious virus. The virus was titered on the rat nephroma cell line for plaque forming units (PFU), which involved developing a new plaquing procedure on this cell line. KRV-DNA synthesis was studied in two ways. First, the effect on production of infectious virions by the addition to infected cells of the DNA inhibitor 5-fluoro-deoxyuridine (FUDR) was determined. Secondly, the comparison in infected and uninfected cells of the incorporation of ^{3}H-thymidine into acid precipitable radioactivity and various times after virion or mock infection of cells was followed. The incorporation of ^{3}H-uridine into RNA and ^{14}C-amino acids into protein was also studied at various times after virion or mock infection of cells.

In order to study the mechanism of KRV DNA synthesis, monolayer rat nephroma cells were infected with KRV and pulse labeled with ^{3}H-thymidine at various times after infection. Alternatively purified virions containing DNA labeled with either ^{14}C or ^{3}H-thymidine were used to infect the cell line. In both cases the cells were lysed at various times after infection and the high molecular weight DNA precipitated in high salt (Hirt procedure). The radioisotope labeled small molecular weight DNA was characterized by its elution from hydroxyapetite columns and by its sedimentation patterns in alkaline and neutral sucrose gradients. The DNA was observed and photographed under the electron microscope. It was also studied for its ability to hybridize on membrane filters with viral and cellular DNA.

An attempt was made to solublize and purify the DNA polymerase found associated with purified KRV. The virions were purified by at least two isopycnic centrifugations in cesium chloride. The enzyme activity was solubilized using sonication of the virion and treatment with the detergent NP-40. The enzyme was purified by successive elution from a DEAE and hybroxyapetite or phosphocellulose columns.

Major Findings:

In order to determine the growth characteristics of KRV in the rat nephroma cell line, duplicate cultures were infected and harvested at various times after infection. There was a latent period of 12 hours during which no virus increases in titer was detected in the fluid medium or within the cells. At about 12 hours an increase in intracellular virus titer was detected. Increasing amounts of virus were formed until 23 hours after infection when no further increase was observed.

The time course of DNA synthesis was observed by adding 5-fluoro-deoxyuridine (FUDR) at various times after KRV infection and measuring the effect of the drug on the final yield of virus. FUDR added up to 8 hours after infection causes complete suppression of virus production. From 8 to 23 hours after infection increasing amounts of virus were formed. Since the final plaque forming units (PFU) represents the viral DNA

synthesized prior to the FUDR addition, viral DNA is synthesized from 8 to 23 hours after infection.

A second method for determining the kinetics of viral DNA synthesis is by following the incorporation of ^{3}H-thymidine into acid precipitable material at various intervals after infection and comparing it to a control (mock infected) culture. Virus infection causes a sharp decrease in cellular DNA synthesis for 6-7 hours when compared to a control culture. After 7 hours, there is an increased synthesis of DNA of about two fold in the infected compared to the control cell. This increase is maintained for the remainder of the virus growth cycle.

The mechanism of KRV-DNA synthesis after infection was studied by infecting the rat nephroma cells with purified virus labeled ^{3}H-thymidine or by short pulses of ^{3}H-thymidine after virus infection. In both cases the small DNA was separated by the Hirt procedure and characterized both on hydroxyapetite columns and on sucrose gradients. Within 1 hour after infection, some of the single stranded linear virion DNA is converted into a double stranded form. After several hours both single and double stranded DNA with the sedimentation characteristics of viral DNA are produced and continue to be produced until the time of cell lysis.

After infection of cells with KRV, protein synthesis was maintained at a rate 75-100% that of the uninfected cell. The rate of cellular RNA synthesis was increased at about 8 hours after infection and was maintained at a rate higher than that of the uninfected cell during the rest of the virus replicative cycle.

The DNA polymerase associated with KRV has been solubilized and purified to a single band on polyacrylamide gels. The enzyme has a molecular weight of 78-80,000. Further characterization of the enzyme will require a batch purification since after solubilization and during purification, the enzyme rapidly loses its stability.

Significance to Bio-medical Research and the Program of the Institute:

Kilham rat virus (KRV) belongs to the parvovirus group which is characterized by small (15-30 nm) virions containing single stranded DNA. The group contains viruses associated with humans (H-1, AAV) as well as rats, (KRV) mice (MVM) cats (feline panleucopenia). KRV was first isolated from a rat sarcoma. It can produce a fatal illness, mental retardation and specific deformaties of growth and development when injected into hamsters. The parvoviruses all contain enough DNA to code for very few proteins. With this small amount of information however, they replicate and cause cell lysis. Little is known about the method of virus control of the cell and virion DNA replication. It may be possible using KRV as a model to determine some of the important aspects of virus-cell interaction.

Proposed Course of the Project:

1. To study further the synthesis of viral DNA in an infected cell. To determine if cellular DNA is completely blocked after infection and to gain some insight into how this control of the cell is exerted.

2. To learn more about the effect of the virus on cellular RNA and protein synthesis. It is possible that the RNA may be made from only one strand of the double stranded replicative intermediate of the virion.

3. To investigate further the DNA polymerase found associated with KRV. The enzyme will be characterized, its requirements for maximal activity determined, and the enzyme products studied. The role of the enzyme in virus replication will be investigated.

Honors and Awards:

Invited to a symposium on Parvoviruses at Putnam Memorial Hospital Institute for Medical Research, Bennington, Vermont, October, 1971.

Invited lecture: Department of Microbiology, Georgetown University, February, 1972.

Elected to membership in the American Society of Biological Chemists.

Publications:

Salzman, L. A., White, W. L., Kakefuda, T.: Linear single-stranded DNA isolated from Kilham rat virus. J. Virology 7: 830-835, 1971.

Serial No. NIAID-84B
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies with Isolated Mammalian Metaphase Chromosomes

Previous Serial Number: Same

Principal Investigators: Dr. Robert S. Lake
Dr. Thomas Y. Shih
Dr. Norman P. Salzman

Other Investigators: None

Cooperating Units: Dr. Karl Piez, NIDR

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	26/12		4/12	30/12
Professional:	16/12		4/12	20/12
Other:	10/12			10/12

Project Description:

Objectives:

To characterize the physical structure and biochemical properties of isolated interphase and metaphase chromosomes which relate to their unique morphologic structure and function in animal cells.

Methods Employed:

Cultured animal cell metaphase chromosomes are isolated from pure metaphase cells by a variety of techniques developed in this laboratory. Chromosomes are further separated from other cellular organelles by differential centrifugation in sucrose or appropriate chemical extraction at acid or neutral pH. Enzymatic activities remaining associated with the chromosomes are selectively extracted and partially purified by ammonium sulfate precipitation, column chromatography and electrophoresis. Protein modification reactions unique to metaphase chromosomes are detected

by analytical gel electrophoresis of histone and non-histone proteins labeled in vivo with radioactive precursors indicative of acetylation, methylation, thiolation or phosphorylation. Assay in vitro of a metaphase chromatin-associated phosphokinase responsible for the transfer of γ-^{32}P from ATP to lysine-rich F1 histone are conducted under conditions found optimal for this enzyme.

The conformation of the basic macromolecular components of isolated Chinese hamster cell chromosomes were investigated with circular dichroism spectra (a direct measurement of optical activity) of chromosomal DNA protein complexes under a variety of experimental conditions. Because of the compositional complexity and large particle size of metaphase chromosomes which complicates the optical measurements, soluble chromatin was isolated from interphase and metaphase chromosomes at low ionic strength. With this material, the basic nucleohistone component could be studied by circular dichroism and thermal denaturation.

Major Findings:

The non-histone chromosomal proteins are found to contain enzymes responsible for histone protein modification reactions. One of these, a lysine-rich (F1) histone phosphokinase was found to be unique in metaphase vs. interphase chromosomes. Its activity as monitored both in vivo, as the existence of a highly phosphorylated F1-histone and in vitro by its ability to transfer γ-^{32}P from ATP to F1-histone is greatly augmented (6-10 fold) in metaphase chromatin. This activation is independent of cyclic nucleotide stimulation and is transient in its appearance in vinblastine or Colcemid arrested mitotic cells.

The circular dichroism (CD) spectrum of the isolated nucleoprotein component of metaphase chromosomes is very similar to that of interphase chromatin. The 275 nm positive CD band of DNA is diminished in magnitude as compared with free DNA, which indicates conformational alteration of DNA in the nucleoprotein complex as found in interphase chromatin. Content of α-helical conformation of chromosomal proteins as revealed by negative CD bands at 222 nm and 209 nm is also similar. In addition, similarity of melting profiles, which indicate the distribution and interaction of proteins with DNA, showed that the basic structure of metaphase and interphase chromosomes is the same. Although there is increased phosphorylation of histone F1 in metaphase chromosomes, no concomitant difference in DNA-conformation has yet been found.

Significance to Bio-medical Research and the Program of the Institute:

An understanding of the physical and biochemical properties of nuclear proteins in metaphase chromosomes will allow rational experimental approaches to the problems of chromosome structure and function. With knowledge of normal chromosomal composition and function, the meaning of chromosomal

aberrations induced by chemicals and viruses can be analyzed.

Proposed Course of the Project:

Examination of the physical and chemical properties of chromosomal constituents will continue. Now that methods are available for the separation of unique chromosomal proteins, emphasis will be placed on determining unique location or function of various proteins in metaphase chromosomes.

Honors and Awards: None

Publications:

Lake, R. S., Goidl, J. A. and Salzman, N. P.: Fl-histone modification at metaphase in Chinese hamster cells. Exptl. Cell Res. In Press.

Serial No. NIAID-84C
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Mechanisms of DNA Replication and Integration

Previous Serial No.: Same

Principal Investigators: Dr. Norman P. Salzman
Dr. George C. Fareed
Dr. Edwin D. Sebring
Ms. Marilyn Thoren

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	62/12			62/12
Professional:	32/12			32/12
Other:	30/12			30/12

Project Description:

Objectives:

To define in molecular terms the mechanism of SV40 DNA replication and the process by which SV40 DNA becomes integrated into the cell genome and to understand mechanisms which control expression of the integrated viral genome.

Methods Employed:

SV40 infected cells have been fractionated to effect a separation of cellular and viral DNA. Radioactive precursors have been incorporated into replicative intermediates (RI's) of SV40. RI has been examined by electron microscopy, isopycnic centrifugation, and velocity rate sedimentation in neutral and alkaline conditions. Immunofluorescent tests have been used to detect the T and V antigens of SV40.

Major Findings:

There is a specific initiation site for the replication of SV40 DNA
Newly synthesized DNA strands have been obtained from replicating molecules by treatment with alkali. These DNA strands have then been sized by velocity gradient centrifugation and used in renaturation studies. It has been observed that the rate of renaturation increases as the size of newly synthesized DNA decreases. These results are those expected if the genetic complexity of the DNA increases as DNA replication proceeds. The relationship has been observed for molecules that are 10 to 90% replicated. These data establish that DNA replication is not initiated randomly, but rather is initiated at a specific site.

The mechanism of replicating SV40 DNA has been further clarified.
Studies have been carried out on (1) the role of the interrupted, circular form of SV40 DNA (DNA II) in replication, (2) determination of a specific origin and termination for replication using bacterial restriction nucleases and (3) the mechanism for SV40 DNA chain growth. Pulse labeled DNA II was isolated and shown to contain the newly synthesized DNA in the interrupted strand. The relative pool size for this intermediate in replication was found to be quite small in relation to the earlier intermediates and indicated that DNA II was not a rate limiting intermediate. In order to define the origin or initiation site for DNA replication young (less than 40% replicated) and mature (greater than 75% replicated) replicating molecules have been isolated and cleaved with the restriction endonuclease of E. coli B. The results demonstrated cleavage occurring only in the unreplicated region of the molecules and an unusual dissociation reaction taking place after cleavage in which the newly synthesized strands separated from the template strands. The results indicate that the site of cleavage may be at either the initiation site or a replication fork and that intact template strands are required at this site in order to stabilize the secondary structure of the replicative intermediate. The mechanism of DNA chain growth has been investigated using very brief periods of pulse labeling. A 4 S DNA chain intermediate has been identified and the results suggest that a discontinuous mechanism is involved in SV40 DNA chain growth.

Significance to Bio-medical Research and the Program of the Institute;

Integration of viral DNA into the cell genome is likely to occur with a number of viruses and is probably important in latent viral infection. These studies hope to explain viral latency and also to ellucidate critical steps in viral transformation by oncogenic viruses.

Proposed Course of the Project:

The role of viral and cellular enzymes in SV40 DNA replication will be defined. The mechanism of initiation of DNA replication will be defined.

Honors and Awards:

Editor, Journal of Virology

Member, Virology Study Section

Gave one-week course entitled "Biosynthesis of Macromolecules," January, 1972, Caracas, Venezuela

Invited lectures at:

1. Columbia University, New York, N. Y.
2. University of Maryland, Baltimore, Md.
3. Fels Research Institute, Philadelphia, Pa.
4. Public Health Research Institute of the City of New York, N. Y., N. Y.
5. University of Illinois School of Medicine, Chicago, Ill.
6. Kansas State University, Manhattan, Kans.
7. University of Connecticut, Storrs, Conn.
8. DNA Replication Meeting, Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.
9. Gordon Research Conference on Cancer

Publications:

Sebring, E. D., Kelly, Jr., T. J., Thoren, M. M. and Salzman, N. P.: Structure of replicating simian virus 40 deoxyribonucleic acid molecules. J. Virol. 8: 478-490, 1971.

Serial No. NIAID-85A
1. Biology of Viruses
2. Biochemical Virology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure of SV40 DNA

Previous Serial No.: Same

Principal Investigators: Dr. Frank DeFilippes

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	22/12			22/12
Professional:	12/12			12/12
Other:	10/12			10/12

Project Description:

Objectives:

Production of defined nucleotide sequences of SV40 DNA and elucidation of their function.

Methods Employed:

Definite double stranded sequences of SV40 DNA are produced by the action of restriction enzymes (deoxyribonucleases) obtained from H. influenzae and H. parainfluenzae. These DNA fragments are separated by electrophoresis and used as templates in RNA synthesis reactions. The in vitro RNA is then tested by hybridization-competition reactions against in vivo RNA from cells which have been exposed to SV40 virus.

Major Findings:

The restriction endonuclease from H. influenzae produces a set of unique fragments of SV40 DNA which sediment between 9s and 4s in neutral sucrose gradients. About 9 of the 11 fragments produced are resolved by electrophoresis in sodium dodecyl sulfate (SDS) buffer in 2.1% polyacrylamide-0.7% agarose gels.

In order to prepare DNA fragments which would be suitable templates for RNA polymerase a detergent-free gel system was developed. The fragments are located after electrophoresis by an ultraviolet scan of the gel and the local region of the gel is cut out and the fragment species extracted from it. Only very small amounts of RNA are produced in the high salt buffer which is generally used to promote selective transcription of intact SV40 DNA. Increased amounts of synthetic RNA, which hybridize to denatured SV40 DNA, are produced in a low salt buffer. All of the synthetic RNA is smaller than tRNA and 20% or more is symmetric. Competition-hybridization experiments show that in vivo late RNA from SV40 infected Vero cells competes with the synthetic RNA made from the 4 largest DNA pieces.

Another restriction enzyme, from Hemophilus parainfluenzae, produces fewer cuts in SV40 DNA and provides larger pieces. A contaminating enzyme activity, which produces acid soluble fragments has hindered work with this enzyme.

Significance to Bio-medical Research and the Program of the Institute:

Production of defined pieces of SV40 DNA may lead to the structure of the genetic element causing malignant transformation of cells.

Proposed Course of the Project:

Restriction enzymes will be used to produce unique fragments of SV40 DNA. The fragments will be used as templates for making synthetic RNA and also for direct hybridization to in vivo RNA obtained from both SV40 lytic and transformed cells. The in vivo RNA will also be used in competition-hybridization experiments against the synthetic RNA. Both approaches are designed to find that region of the viral genome which is responsible for oncogenic transformation.

Honors and Awards: None

Publications: None

Serial No. NIAID-87A
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Properties of the Nucleic Acid and Protein Components of Adenovirus-Associated Viruses

Previous Serial Number: Same

Principal Investigator: Dr. James A. Rose

Other Investigators: Dr. Barrie J. Carter
Dr. George C. Lavelle
Mr. Frank Koczot

Cooperating Units: Dr. Raphael Dolin, L.I.D.; Dr. Jacob V. Maizel, Jr., Albert Einstein College of Medicine, New York, N.Y.;
Man Years: Dr. Aaron J. Shatkin, Roche Inst., Nutley, N.J.

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	31/12		12/12	43/12
Professional:	9/12		12/12	21/12
Other:	22/12			22/12

Project Description:

Adenovirus-associated viruses (AAV) are small, DNA-containing viruses that multiply only in cells which are co-infected with an adenovirus. Herpesviruses also provide some function(s) required for AAV multiplication; AAV antigen(s) is synthesized but an increase in AAV infectivity or particles has not been observed. Four AAV serotypes are now known. We have shown previously that AAV DNA is segregated in a way not as yet observed with any other virus. Single, complementary DNA strands are separately encapsidated in different virions. On extraction of viral DNA, the complementary strands anneal to form duplex molecules. There are two species of complementary DNA strands which can be separated in CsCl density gradients. Studies are directed toward the biological and biochemical characterization of AAV infection.

Objectives:

1. To analyze the structure and function of the complementary strand species of AAV-DNA.

2. To identify the helper virus-mediated step(s) in the AAV replication sequence.

3. To analyze the structure of in vivo synthesized AAV RNA induced by either partial or complete helper viruses.

Methods Employed:

The AAV are produced in KB cells in spinner culture. Viruses are purified in CsCl density gradients, and DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodecyl sulfate and phenol. The complementary strand species are separated by centrifuging denatured, bromodeoxyuridine-substituted DNA to equilibrium in neutral CsCl density gradients. Viral RNA synthesized in vivo or in vitro is labeled with ^{3}H or ^{32}P. For base composition determinations, ^{32}P-labeled DNA and RNA are hydrolyzed, and the mole fraction of each base calculated from high voltage electrophorograms. DNA/RNA complementarity is determined by nucleic acid hybridization and hybrids are detected on nitrocellulose filters or in CsCl density gradients. RNA is further analyzed by sedimentation in dimethyl-sulfoxide sucrose gradients.

Major Findings:

1. The two complementary strand species of 5-bromodeoxyuridine-substituted, adenovirus-associated virus type 2 (AAV-2) deoxyribonucleic acid were preparatively separated in CsCl density gradients and further purified by sedimentation through 5 to 20% sucrose. The base composition of each strand species was determined, and it was found that the species banding at a greater density in CsCl (heavy strands) had an expected higher thymidine content (26.5%) than that (21.7%) of the less dense species (light strands). Furthermore, The base composition of in vivo-synthesized, AAV-specific ribonucleic acid was similar to that of light-strand deoxyribonucleic acid, and this ribonucleic acid apparently hybridized only with heavy strands. These observations indicate that the heavy-strand species alone serves as the transcriptional template in vivo. This study represents the first instance in which the base composition and specificity of in vivo transcription have been determined for each of the complementary strands of an animal virus deoxyribonucleic acid.

2. The structural proteins of adenovirus-associated virus (AAV) types 1, 2, and 3 were analyzed by acrylamide gel electrophoresis. In each case, one major protein (C) and two minor proteins (A and B) were identified. Component C had an estimated molecular weight of 62,000 daltons, and the molecular weights of components A and B were found to be 87,000 and 73,000 daltons, respectively. Coelectrophoresis of adenovirus and AAV proteins revealed an overlap only between the adenovirus fiber-penton component and the AAV C polypeptide. Among AAV serotypes, homologous components were electrophoretically identical, except that the C component of AAV-2 was of slightly lower molecular weight than the C components of AAV-1 and AAV-3. The relative incorporation of ^{14}C-arginine and ^{14}C-mixed amino acids into the three polypeptides of

AAV-2 was similar, indicating an absence of an arginine-rich component. In addition, AAV-2 was found to have a substantially lower arginine content than helper adenoviruses.

3. The adsorption of adenovirus-associated virus type 3 (AAV-2) by KB cells and the subsequent penetration of the AAV genome to the cell nucleus was measured with and without helper adenovirus type 2 (Ad 2). It was found that the helper virus did not enhance either process. On the other hand, a synthesis of AAV deoxyribonucleic acid (DNA) or ribonucleic acid (RNA) was not detected by nucleic acid hybridization after KB cells were infected with AAV-2 alone, whereas both AAV DNA and RNA synthesis were readily detected when cells were additionally infected with Ad 2 or herpes simplex virus type 1, a partial helper of AAV replication. AAV RNA synthesis was initially observed 10-11 hr following similtaneous infection with Ad 2, but the interval between AAV infection and AAV transcription could be reduced to 4-5 hr when cells were first infected with Ad 2 for 10 hr. It was estimated that AAV DNA synthesis accounted for 3% of the total DNA in cells after a simultaneous infection with Ad 2. These findings, together with the previous observation that adenovirus provides a helper function(s) after AAV uncoating, suggest that AAV are defective only subsequent to the uncoating process, and that helper viruses may provide a factor (s) needed for initiating synthesis of AAV DNA and/or RNA.

4. We have analyzed RNA synthesized in KB cells co-infected with adenovirus-associated virus type 2 (AAV-2) and either adenovirus type 2 (Ad 2) or herpes simplex virus type 1 (HSV-1). Using either type of helper virus, synthesis of AAV RNA was readily detected by DNA/RNA hybridization. As is the case for AAV RNA synthesized with helper Ad 2, the AAV RNA synthesized with HSV-1 as helper annealed only to the thymidine-rich (minus) AAV DNA strand. In addition, AAV RNA synthesized with either type of helper (i) contained similar nucleotide sequences as determined by hybridization inhibition tests and (ii) had a mean molecular weight of approximately 7.5×10^5 based on sedimentation in dimethyl-sulfoxide sucrose gradients. These experiments suggest that the restricted helper function of HSV-1 is not due to abnormal transcription of the AAV genome. Since the mean molecular weight of AAV RNA is equivalent to 40-50% of the AAV genome, as few as 2-3 AAV RNA species may be transcribed in vivo. In contrast with adenovirus RNA, cleavage of AAV RNA following transcription was not observed.

Significance to Bio-medical Research and the Program of the Institute:

Human infection with AAV has been shown to be frequent, but its significance is not yet known, since a virus-specific clinical disease has not been defined. Characterization of biochemical and biological properties of these viruses may be important with respect to human disease, because 1) although now inapparent, they might be human pathogens and, 2) the mechanism(s) by which helper viruses assist AAV replication are relevant to understanding natural modes of viral infection and may be important with respect to the development of biochemical controls of virus replication.

Proposed Course of the Project:

Continue to study AAV replication and defectiveness.

Honors and Awards:

Invited lectures at:
1. University of Maryland, College Park, Md.
2. Cold Spring Harbor Lab., Cold Spring Harbor, N. Y.
3. Second International Congress for Virology, Budapest, Hungary.

Publications:

Rose, J. A. and Koczot, F.: Adenovirus-associated virus multiplication VI. Base composition of the deoxyribonucleic acid strand species and strand-specific in vivo transcription. J. Virol. 8: 771-777, 1971.

Rose, J. A., Maizel, Jr., J. V., Inman, J. K. and Shatkin, A. J.: Structural proteins of adenovirus-associated viruses. J. Virol. 8: 766-770, 1971.

Rose, J. A. and Koczot, F.: Adenovirus-associated virus multiplication VII. Helper requirement for viral DNA and RNA synthesis. J. Virol. In Press.

Carter, B. J. and Rose, J. A.: Adenovirus-associated virus multiplication VIII. Analysis of in vivo transcription induced by complete or partial helper viruses. J. Virol. In Press.

Serial No. NIAID-87B
1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Properties of Adenovirus DNA

Previous Serial No.: Same

Principal Investigators: Dr. James A. Rose
Dr. Claude F. Garon

Other Investigators: Ms. Karen W. Berry
Mr. James W. Garrison

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	48/12			48/12
Professional:	14/12			14/12
Other:	34/12			34/12

Project Description:

Physical and biochemical characterization of the adenovirus genome.

Objectives:

Application of physical and biochemical techniques for analyzing structure and function of the adenovirus genome.

Methods Employed:

Viruses are produced in KB cells and purified in CsCl equilibrium density gradients. Viral DNA is extracted from purified virus by treatment with proteolytic enzymes, sodium dodecylsulfate, and phenol. Structural properties of adenovirus DNA are studied by zonal sedimentation and electron microscopic analysis following treatment with nucleases and/or denaturation and renaturation. Electron-microscopic mapping techniques are used to identify regions of difference and similarity between genomes from selected virus serotypes.

Major Findings:

Human adenoviruses contain double-stranded, linear DNA with a molecular weight of 20-25 x 10^6 daltons. Because native DNA molecules do not form circles on annealing after treatment with E. coli exonuclease III, others have concluded that the adenovirus genome does not contain terminally repetitious nucleotide sequences. However, our electron microscopic studies with DNA from several adenovirus serotypes reveal that single-stranded, circular molecules are formed when native adenovirus DNA is denatured and then annealed. These single-stranded circles do not form if a limited number of nucleotides (3-5%) are removed from the 3' ends of the native molecules by exonuclease III digestion prior to denaturation and annealing. We therefore conclude that adenovirus DNA does have terminally repeated sequences, but these sequences are inverted. As a result, each DNA strand contains complimentary, terminal sequences which may self-anneal to form the observed single-stranded circles.

Significance to Bio-medical Research and the Program of the Institute:

Further definition of structural and functional relationships of adenovirus DNA may lead to some understanding of the molecular basis of viral carcinogenesis and add to knowledge of the interactions between virus and cell during viral infection.

Proposed Course of the Project:

1. Continue to analyze the structure of viral DNA by biochemical and electron-microscopic techniques.

2. Investigate the genetic functions of physically defined segments of viral DNA.

Honors and Awards: None

Publications:

Kelly, Jr., T. J. and Rose, J. A.: SV40 integration site in an adenovirus 7-SV40 hybrid DNA molecule. Proc. Natl. Acad. Sci. 68: 1037-1041, 1971.

Serial No. NIAID-87C

1. Biology of Viruses
2. Molecular Structure
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Properties of Herpesvirus DNA

Previous Serial No.: Same

Principal Investigator: Dr. James A. Rose

Other Investigator: Dr. Alan S. Rabson, Lab. of Pathology and Biology, N.C.I.

Cooperating Units: Dr. Robert W. Chambers, Dep't. of Pathology, Georgetown University Medical School

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	14/12			14/12
Professional:	4/12			4/12
Other:	10/12			10/12

Project Description:

Physical and biochemical characterization of herpesvirus genomes.

Objectives:

1. To improve techniques for production and purification of herpesviruses.

2. To assess genetic relatedness among human herpesviruses.

Methods Employed:

Viruses are produced in HEP2 and WI-38 cells and concentrated by differential or continuous flow centrifugation. DNA is recovered from virus preparations by treatment with proteolytic enzymes, sodium dodecylsulfate (SDS) and phenol. Further purification of viral DNA is achieved in CsCl density gradients. Assessments of genetic relatedness are based on DNA/DNA and DNA/RNA hybridization reactions utilizing nitrocellulose filters.

Major Findings:

Techniques for production and purification of herpesviruses have been improved. High-titered yields of human cytomegalovirus (CMV), strain AD 169, were produced in WI-38 cells in large roller bottles. The production of high-titered yields of a human CMV strain has not been reported previously. The use of roller cultures and a relatively high infection multiplicity (0.1 PFU/cell) were required for increased virus yields. Maximum plaque titers were observed by the 4th day after infection at which time infectivity in the medium was 200 times greater than that associated with the cells. Virus released into the medium was recovered by sedimentation in a sucrose gradient in a continuous-flow centrifuge rotor. Maximal viral infectivity was found at a sucrose concentration of 42%, equivalent to a density of 1.18 g/cm^3. Deoxyribonucleic acid extracted from these preparations was about 80% viral and 20% cellular as judged by equilibrium centrifugation in cesium chloride density gradients.

Significance to Bio-medical Research and the Program of the Institute:

Certain herpesviruses are known to be important human pathogens. In addition, there is evidence that the herpes-like particle (EB Virus) which has been associated with human lymphoma cells is related to the infectious mononucleosis agent. Whether or not EB virus is an etiologic agent for human lymphomas is not known. Further definition of structural and functional properties of herpesvirus DNA may lead to some understanding of the mechanism of chronic viral infection as well as to enhance present capabilities for analyzing the possible role of these viruses in human oncogenesis.

Proposed Course of the Project:

1. Continue genetic relatedness studies among members of the herpesvirus group.

2. Relate genetic function to physically defined regions of viral DNA.

Honors and Awards: None

Publications:

Chambers, R. W., Rose, J. A., Rabson, A. S, Bond, H. E. and Hall, W. T.: Propagation and purification of high-titer human cytomegalovirus. Appl. Microbiol. 22: 914-918, 1971.

Serial No. NIAID-88
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Synthesis of Proteins in SV-40 Infected Cells

Previous Serial Number: Same

Principal Investigator: Dr. Stanley Barban

Other Investigators: Mr. Henry Schulze

Cooperating Units: Dr. Ettore Appella, N.C.I.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	34/12			34/12
Professional:	12/12			12/12
Other:	22/12			22/12

Project Description:

Objectives:

1. To elucidate the role of the viral genome in directing the synthesis of both viral and non-structural proteins in productive and transforming infection.

2. To compare the amino acid composition of SV40 mutant strain which differ in plaque-type and other biological properties and relate these properties to the physical and chemical composition of the capsid proteins of the virions.

Methods Employed:

SV40 virus was grown in primary and continuous African green monkey kidney cells and purified by a sequence of CsCl density gradient and equilibrium centrifugations. Radioactively labeled virus was prepared by propagating the virus in media containing ^{3}H or ^{14}C amino acid mixtures. Fractionation of purified virions into subunits was performed after dissociation of the virions and separation of macromolecules by zonal centrifugation gel electrophoresis, column chromatography and isoelectric focusing in gels or density gradient columns, and peptide mapping of tryptic digests

of the viral subunits. Specific viral proteins were further identified by immunological methods.

Major Findings:

Electrophoretic differences in the capsid proteins of SV40 plaque size mutants. We have previously shown that SV40 virions contained at least four polypeptide components. The major structural component was the capsid protein comprising over 75 per cent of the total virion. Comparison of the capsid proteins of the large and small plaque SV40 mutants by the isoelectric focusing technique revealed a distinct difference in their isoelectric points. By this technique, it has also been possible to distinguish the capsid proteins of a minute-plaque mutant from the large-plaque type SV40 mutant. In contrast, the capsid proteins of the small and minute plaque SV40 strains appear to be generally similar, and thus far cannot be distinguished by their isoelectric points.

Peptide differences of the capsid proteins of SV40 mutants. Comparative studies employing two-dimensional peptide mapping of the tryptic digests of the capsid proteins from the small and large plaque type SV40 mutants have revealed a significant difference in their peptide composition. These results offer an explanation for the difference in the electrophoretic pattern of the capsid proteins.

Significance to Biomedical Research and the Program of the Institute:

Since the activity of one or more viral genes seems to be required to induce and maintain the transformed state, identification and characterization of SV40 virus specific proteins is of basic importance for studies on the mechanism of virus-induced cell transformation. It also has obvious basic and practical significance to problems of control mechanisms of virus induced host cell allerations. The findings of a difference in the structural composition of SV40 mutant strains with different biological properties is of importance in genetic and immunological studies of mutant tumor viruses.

Proposed Course of the Project:

Efforts will be made to further characterize the peptides and constituent amino acids of the coat proteins of the SV40-plaque mutants and to relate the structural differences with differences in their biological properties. Additional SV40 mutants will be examined to determine whether structural viral differences can be correlated with their biological properties. The synthesis of SV40 specific proteins and macromolecules will be examined at different times following infection and comparisons made using the mutant strains.

Honors and Awards: None

Publications: None .

Serial No. NIAID-88B
1. Biology of Viruses
2. Physical Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure and Activity of Isolated Chromatin

Previous Serial Number: None

Principal Investigators: Dr. Thomas Y. Shih
Dr. Malcolm A. Martin

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	14/12		8/12	22/12
Professional:	4/12		8/12	12/12
Other:	10/12			10/12

Project Description:

Objectives:

To study the structure and template activity of chromatin isolated from virus-transformed or phenotypically normal cells in order to further understand the mechanisms regulating expression of integrated viral genomes.

Methods Employed:

An SV40 transformed cell line (SV-T2), known to contain viral-specific RNA was employed for the present study. A quantitative procedure for isolation and purification of chromatin from tissue culture cells was developed. This procedure involves the homogenization of cells in saline-EDTA (0.075M NaCl, 0.024M EDTA, pH 8.0), in which chromatin is not soluble, several differential centrifugations to remove other cell components, and finally, sedimentation through a dense sucrose solution. The purified chromatin was analyzed for its chemical composition of DNA, by the diphenylamine method, protein, by Lowry's method, and RNA, by the orcinol reaction. Chromatin was further characterized

by its ultaviolet absorption spectrum and thermal denaturation profiles. Template activity of chromatin was assayed in DNA-dependent RNA polymerase system. The exogenous RNA polymerase added was purified from E. coli B according to the procedure of Chamberlin and Berg to the step of Fraction 4. The RNA synthesized in vitro with purified chromatin as template was isolated by DNase digestion and phenol deproteinization. The nature of the in vitro RNA sequences was studied by molecular hybridization. Viral specific RNA was detected by measuring the effect of the in vitro RNA on the reassociation of P^{32}-labeled SV40 DNA.

Major Findings:

In order to study the template activity of chromatin and to compare the RNA product made in vitro with the chromatin template with the in vivo RNA, it is important that the isolated chromatin resemble the intracellular chromatin as much as possible; the extraction should be carried out to eliminate conditons which alter DNA-histone interaction. 75% of cell DNA was recovered as purified chromatin as determined by diphenylamine method. Thus it seems likely that all of the chromatin DNA is represented in the isolated material. The isolated chromatin possesses a chemical composition in terms of mass ratio of DNA:protein:RNA of 1.0:2.5:0.07. The ultraviolet absorption spectrum shows a maximum at 257.5nm and A_{230}/A_{260} ratio of 1.0 which is consistent with the protein/DNA ratio determined by chemical methods. The melting profiles of the isolated chromatin in 2.5 x 10^{-4}M EDTA, pH 8.0 exhibited at least four overlapping transitions which are most clearly revealed by plotting the slope of the melting profiles against temperature. There are two major peaks at 77°C and 71°C which represent stabilization of DNA due to histone-binding and two minor peaks at 63°C and 48°C which represent stretches of DNA possibly bound with non-histone proteins and free DNA segments, respectively. Purified cellular DNA had a T_m of 47°C under these experimental conditions. The template activity of chromatin as assayed by measuring the initial rate of RNA synthesis in the presence of constant amount of RNA polymerase is 20% that of purified transformed cell DNA.

Experiments are currently in progress comparing the RNA prepared in vitro using the chromatin template, with in vivo RNA purified from cells growing in culture with special emphasis on the SV40 specific RNA in each case. We are also examining the properties of cellular RNA that appear in vitro and in vivo.

Significance to Bio-medical Research and the Program of the Institute:

The regulation of gene expression in eukaryotes undoubtedly involves an interaction of cellular DNA and its associated proteins. There is evidence that the chromosomal DNA complexed with histone, the basic chromosomal protein, is unable to function as template for RNA synthesis. Therefore it is implied that histone plays a role in the regulation of the read-out of the potential genetic information. The transcription of integrated genomes of tumor viruses may be also regulated by cellular

mechanisms. Since it has been shown that a spectrum of virus DNA transcription can be measured when several different transformed lines are examined and, in the case of the abortive transformants, no expression of SV40 genetic information occurs. The present study is directed to address to these problems by studying the biochemical properties of isolated chromatin.

Proposed Course of the Project:

The nature of the RNA generated with chromatin template in the DNA-dependent RNA polymerase reaction will be further examined by molecular hybridization in order to reveal the transcriptional control by chromosomal proteins.

Honors and Awards: None

Publications: None

Middle America Research Unit

Balboa Heights, Canal Zone

Summary 1

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PHS-NIH
Summary Statement
Middle America Research Unit
Balboa Heights, Canal Zone
July 1, 1971 through June 30, 1972

Karl M. Johnson, M.D., Director

After 14 years of operation, this laboratory was informed in November 1971 that NIH intended to close it at the end of the current reporting period. In December Dr. K. M. Johnson was recipient of the Bailey K. Ashford award given every three years by the American Society for Tropical Medicine. In March 1972 NIH announced that the scientific programs of the laboratory would be supported for one or two more years through a contract with the Gorgas Memorial Institute. This, then, represents the last MARU annual report.

Research programs conducted by the laboratory during the past year were focused on diseases caused by certain arboviruses, arenaviruses, and on hepatitis. Work of the associated U.S. Army research unit concentrated on leishmaniasis. In all cases primary emphasis was given to attempt to elucidate the natural history of infection both by field studies and investigations of the biological behavior of representative viruses in laboratory model systems.

Research Accomplishments

Tacaribe Viruses.

A new outbreak of Bolivian hemorrhagic fever with hospital contact infections. The virus which caused the peculiar, devastating outbreak of hemorrhagic fever with hepatic insufficiency in Cochabamba, Bolivia early in 1971 was finally identified as Machupo virus. This strain, which appears to have emanated from a here-to-for disease-free area of the country, produced more secure human disease than the prototype and was highly communicable from person to person; new and dangerous behavior for this pathogen. (Peters)

Can Machupo virus induce lymphoma in man? A search for late effects of Machupo virus infection of man was made in Bolivia, prompted by the diagnosis of a bizarre lymphosarcoma of the thymus in a staff scientist eight years after primary infection. Although no direct evidence for malignant sequela was obtained, less than half of the proven patients were located. Nevertheless, a cohort of former patients and controls was established for future examination, and many persons were found who still had virus-specific neutralizing antibodies in concentrations high enough to make feasible the preparation of specific gamma globulin. (Webb, Mackenzie)

Biological control of hemorrhagic fever by rodent reservoir replacement found not feasible. By use of two distinct colonies of Calomys callosus derived from areas where Machupo and Latino (a human non-pathogenic Tacaribe complex agent) viruses were endemic, it was shown that a) the phenomenon of chronic viremic infection with anemia and splenomegaly

was specific for Machupo virus, b) that the "fit" between virus and host was at least partially host-specific since some rodents from the Latino colony made antibody to and cleared Machupo virus from the circulation and c) that biological substitution of Latino Calomys for the race already found in the disease-endemic region would probably not work since many of the former animals did display chronic infection with viremia and viruria. (Webb, Walton, Wiebenga)

Attenuated strain of Machupo virus raises hope for vaccine development. Serial passage of Machupo virus in suckling, specific pathogen-free mice resulted in alteration of several biological properties. Beyond 20 passages guinea pigs were not killed and marmosets failed to exhibit viremia. Many of the latter animals survived and were clinically immune to challenge by virulent low passage virus three months later. After 40 passages there was no evidence for virus replication in two types of cultured human cells. Pathogenicity for mice and hamsters was retained through 80 passages. These results point the direction for future work on production of a Machupo virus vaccine. (Justines, Johnson)

Venezuelan equine encephalitis (VEE)

Phenotypic markers indicate virus virulence for equines. Virus plaque size and pH dependence of hemagglutinin were thoroughly confirmed as subtypic markers correlating with virus virulence for equines. It was shown that plaque size of virulent strains was influenced by sulfated polysaccharides. Several other markers were sought without success. (Martin, Eddy)

Antigenic properties of viruses found to correlate with equine virulence. The patterns of clinical and virological response to several VEE subtypes were elucidated in experimentally infected horses. Avirulent strains induced little viremia, little or no clinical illness beyond fever, and generally elicited modest movements of antibody with very low titers of complement-fixing substances. In contrast, virulent strains produced high viremia (6-8 dex/ml) between the first and fourth day post inoculation. Virus and increased protein were found in cerebrospinal fluid at the time of peak viremia. Neutralizing antibodies usually appeared about day 5 and were temporarily correlated with onset of clinical encephalitis. Complement fixing antibodies were not seen until day 6 or 7. Antibody titers reached very high levels by 9-10 days. Homologous responses to the antigenic subtype administered were almost always higher than to heterologous strains suggesting that such sera may prove useful in refining present antigenic classification of members of the VEE complex. (Walton, Dietz)

Vesicular stomatitis virus (VSV).

Biological specificity of transovarial transmission by phlebotomine flies elucidated. Transovarial transmission of the Indiana serotype was found to be species-specific in Phlebotomus flies. Both L. trapidoi and L. ylephiletrix were capable of such transmission, while L. gomezi and L. sanguinaria, also anthropophilic in feeding habits were not. Transmission by L. trapidoi was successfully demonstrated through two consecutive generations

with suggestive evidence for an increase in efficiency of the mechanism in the F_2 females. Detailed investigation of possible effects of virus infection on longevity and reproductive capacity of infected females disclosed no obvious differences from uninfected controls. (Tesh, Chaniotis)

Studies of phlebotomine biology are useful in understanding and control of associated infectious diseases. Further work was done on the biology of L. trapidoi. It was found that fructose and sucrose were the prefered sugar sources and that several others were rejected completely. Flight range of adult females was highly restricted, probably not exceeding 100 meters, although vertical movement to the canopy of large trees was observed at night, together with descent to the base of such trees where resting positions were taken up during the day. Breeding site of this species was localized to the forest floor, was continuous, and was extensive rather than focal. Variations in density of breeding appeared to correlate with micro-variation in accumulation of leaf litter and small currents of runoff of surface water. (Chaniotis, Rutledge)

Other sandfly-associated viruses.

New viruses discovered; several are probably transmitted transovarially. More than 200 agents were recovered from sandflies. Some have been identified as Changuinola viruses. All others appear to belong to about five serotypes (four of which are new) of the Phlebotomus fever group. All isolates of Changuinola virus were from female flies, whereas Phlebotomus fever agents were recovered with nearly equal frequency from males and females, strongly suggesting that these viruses, like VSV-Indiana, may prove to be transovarially transmitted. New viruses have been immunologically characterized, a large effort that required a standardized re-examination of all known agents of the group, and reagents were prepared in anticipation of future seroepidemiological studies. (Tesh, Peralta)

Group B arboviruses

Central America found highly vulnerable to epidemics of dengue and yellow fever. A microplaque reduction test was successfully applied for detecting specific infections in human sera from Central America. More than 15,000 individual determinations were made with this economical procedure. It was found that antibodies to yellow fever virus were common in persons alive at the time of the last epizootic in the region, 1948-56. That these were largely due to vaccine was supported by an equal sex distribution, a relative plateau in frequency among adults, and the general absence of such antibodies in El Salvador, the only country spared during the epizootic. Antibodies to dengue type 2 virus were common among persons born prior to 1942, the date of the last known epidemic in the region. They were localized to lowland towns on or near the seacoast. Evidence for past activity of type 3 and possibly type 1 virus was also found, but not for type 4. Low level continuous endemic behavior of St. Louis encephalitis and Ilheus viruses was documented. (Peralta, Johnson)

Hepatitis

Au associated hepatitis more severe than non-Au disease in adults. Clinical patterns of hepatitis with and without detectable Australia antigenemia (Au) were compared among adults. Au-positive patients had more insidious onset, less fever but more severe chemical abnormality than did those without antigenemia.

Immunoglobulin levels discriminate Au-positive and Au-negative disease. The inverse correlation between presence of Au in blood and elevation of total IgM previously found was confirmed. A subgroup of patients was encountered, however, which had neither Au nor increased IgM. Antibody tests to Au are planned to see whether these are really Au infections or whether they represent hepatitis caused by one or more unusual agents. (Peters, Reeves)

Infection and chronic antigenemia rates differ in three Indian populations. Chronic Au antigenemia was found to vary significantly among different Indian groups. When correlated with age-specific prevalence of antibody to Au the data led to the conclusion that Guaymi Indians experience a much higher frequency of chronic Au infection than do Choco Indians. A third tribe, the San Blas Cuna, had less infection than either of the other two groups and no evidence of chronicity. These data and the relative accessibility of the distinct populations provide exciting opportunities for further elucidation of the epidemiology of the Au agent and of the factors responsible for chronicity of infection. (Reeves, Peters)

Leishmaniasis

Cotton rat proves excellent model for dermal leishmaniasis. More than 30 new cases of the disease were documented, most of them among soldiers exposed during training exercises. Several of these strains induced impressive ulcerative lesions in cotton rats Sigmodon hispidus which are being developed as an experimental model for study of pathophysiology of infection.

Study of cellular immune function in human leishmaniasis initiated. A project to assess humoral and cellular immune responses in patients was initiated. Very preliminary findings suggest disassociation of macrophage inhibition and lymphoblast transformation activities. Experimental skin test antigens are being prepared. The eventual goal is to see whether there is a scientific basis for consideration of administration of transfer factor to some patients. Such a possibility is viable since about 25% of patients relapsed after apparently successful treatment with antimonial drugs.

Serial No. NIAID-100-A
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Surveillance of Infectious Disease Situations to Determine Investigative Potential for MARU

Previous Serial Number: Same

Principal Investigators: Dr. K. M. Johnson and Lt. Col. J. C. Burke

Other Investigators: Dr. P. H. Peralta, Dr. P. H. Webb, Dr. R. B. Tesh, Dr. T. E. Walton, Lt. Col. G. A. Eddy, Dr. D. H. Martin, and Dr. W. C. Reeves

Cooperating Units: Ministries of Health in Panamá, Guatemala, Bolivia, Ecuador, Honduras, Nicaragua and Costa Rica.
PAHO/PASB/WHO, Zone III Office, Guatemala
Health Director, Canal Zone
Office of the Chief Surgeon, USARSO, Canal Zone
Gorgas Memorial Laboratory, Republic of Panamá
Canal Zone Military Dispensaries (U.S. Army, Navy, and Air Force)
Hospital del Niño, Republic of Panamá
Hospital Division, Chiriqui Land Company, Panamá
Hospital Roosevelt, Guatemala
Laboratorio de Salud Publica, Nicaragua
Hospital Infantil Maria Teresa, Guatemala
Laboratorio Veterinario, Ministry of Agriculture, Republic of Panamá

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	1 2/12		4/12	1 6/12
Professional:	6/12		4/12	10/12
Other:	8/12			8/12

Project Description:

Objectives:

1. To provide for prompt recognition of changes in human or animal

morbidity and mortality which might lead to further investigations of research interest:

a. By prospective surveillance programs.
b. By investigation of individual cases or epidemics in progress in the field.
c. By retrospective serum surveys of human and animal populations.

2. To maintain contacts with various health agencies in the Canal Zone, Republic of Panamá, and throughout Central America in order that promising situations may be learned of and investigated in the shortest possible time.

3. To gather sufficient information concerning an epidemiologic situation to:

a. Pursue studies in depth as a separate project.
b. Refer to some other agency
c. Drop from consideration.

Methods Employed:

Prospective studies include clinical surveillance for cases of acute neutropenia at Gorgas Hospital, and the collection and testing of cord blood samples obtained from several local hospitals for immunoglobulin levels.

When leads of importance are obtained in the course of testing clinical case specimens or through information supplied by cooperating agencies, arrangements for field activities are made. In the Canal Zone, these are made through the Canal Zone Health Bureau and Gorgas Hospital; contacts are also maintained with the Office of the Chief Surgeon, U.S. Army Southern Command, and clinical facilities of individual military units. Throughout Central America and Panamá, contacts with individual Health Ministries are initiated and maintained with the cooperation of the Pan American Health Organization Zone III Office in Guatemala.

Laboratory procedures include attempted isolation of the agents involved by inoculation of material into appropriate cell cultures and laboratory animals. Standard serologic procedures including in some cases fluorescent antibody techniques are used to test for specific serum antibodies.

Field procedures include specialized epidemiologic, clinical, entomologic, and biologic techniques.

Major Findings:

The year was marked by few epidemic notifications. Small outbreaks of poliomyelitis (Type 1) and A_2 influenza were detected in Panamá. The study of neutropenia in children seen at Gorgas Hospital was completed. A total of 40 cases was accumulated, and although work continues, no infectious agent has yet

been isolated. Nevertheless, more than half of the patients were seen in the months of December-March, a fact which, in the absence of an increase in total patients attending clinics during this season, is strongly suggestive of an infectious etiology. Neutropenia did not persist beyond four weeks in any instance, and the great majority of patients experienced mild respiratory or gastrointestinal illnesses with no other distinguishing clinical features. Whether a specific etiologic agent or individual host response to a variety of microbes best explains the observations is not clear at present.

Significance to Bio-Medical Research and the Program of the Institute:

The project has been the simple cornerstone of nearly every significant research program conducted by this laboratory. In view of the foreseeable termination of the laboratory, it will be abandoned on the reasoning that nothing can be done about new opportunities that it might uncover.

Serial No. NIAID-100-B
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Tacaribe Group of Viruses

Previous Serial Number: Same

Principal Investigators: Dr. P. A. Webb, Dr. K. M. Johnson, and Dr. G. J. Justines

Other Investigators: Dr. N. H. Wiebenga, Dr. C. J. Peters, Dr. T. E. Walton, and Mr. J. Vogel

Cooperating Units: Rockefeller Foundation, Cali, Colombia; Dr. R. B. Mackenzie
Channing Laboratory, Boston, Mass; Dr. N. A. Young
USAMRU Panama; Major L. J. Ackerman
Viropathology Unit, Center for Disease Control, Atlanta, Georgia; Dr. Frederick A. Murphy
U.S. Army Medical Research Institute of Infectious Diseases, Frederick, Maryland; Mr. R. W. Kuehne
Instituto Nacional de Enfermedades Transmisibles, Ministerio de Salud, La Paz, Boliva; Dr. R. Mercado
Instituto Nacional de Laboratorios de Salud, La Paz, Bolivia; Dr. L. Valverde Chinel
NIAID, Rocky Mountain Laboratory, Hamilton, Montana; Dr. John Coe

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	6 9/12			6 9/12
Professional:	2 6/12			2 6/12
Other:	4 3/12			4 3/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PH43-67-59	K.M. Johnson	Smithsonian	C.O. Handley, Jr.	$6,720

Project Description:

Objectives:

1. To characterize and determine the natural relationship of viruses of the Tacaribe group by study of their immunological, morphological, and biophysical properties, the bionomics and pathogenesis in animals, particularly in the natural rodent reservoir of Machupo virus, Calomys callosus.

2. To develop a safe, effective vaccine for Bolivian hemorrhagic fever.

3. To continue limited routine epidemiological surveillance in certain areas of the Department of Beni, Bolivia, and to study more intensively areas where evidence of Machupo virus activity is high.

4. To make available reagents and provide facilities for diagnosis of viruses in this group to other North and South American investigators and to assist them in active control measures to contain new outbreaks of human disease.

Methods Employed:

Standard virus isolation and serologic techniques were employed, principally the use of cell cultures, fluorescent antibody determinations, immunoelectrophoresis, animal inoculation, and standard pathologic sectioning and staining. Virus-infected organs were sectioned and fixed at MARU and submitted to the Channing Laboratory for detailed pathologic studies, and glutaraldehyde-fixed cell cultures were sent to CDC for electron microscopic study. Field collections were made in Yacuma Province, Department of Beni, Bolivia, where an outbreak of hemorrhagic fever (HF) was recorded for the first time. Long-term ($\pm$ 8 years), follow-up studies on previously documented H.F. cases and control cases were made in San Joaquin, Beni, Bolivia, and its immediate environs. Follow-up bloods were collected from Cochabamba contacts and from the single survivor case. Mammal skins and skulls were submitted to the U.S. National Museum, Smithsonian Institution, for identification.

Major Findings:

Epidemiology of BHF: The Cochabamba outbreak was found to be due to Machupo virus. This outbreak occurred between January to March, 1971, and consisted of six cases with five deaths, three patients having evidence of hepatitis not previously documented in this disease. Follow-up sera on all contact cases contained no Machupo antibody (CF or N) and no further cases have been reported from Cochabamba. The source of infection of the index case was not found. She had been vacationing in a settlement near Santa Ana, Yacuma Province, Department of Beni, at least 50 miles outside the known HF area. Trapping in and around the house she visited yielded Zygodontomys but no Calomys rodents. No virus was isolated from the tissues of these animals. No other illness occurred in her contacts in this area, nor did their sera contain Machupo antibody. It was

considered unlikely that she acquired her infection in that area. However, in November 1971, the first notification of HF-like illnesses and deaths were reported to La Paz from the Santiago area of Yacuma Province, to the north of Santa Ana. Upon investigation, it seemed probable that the index case, who survived and had Machupo N antibody, occurred in August 1971. Three subsequent cases were fatal. Virus was isolated from the spleens of two of these patients and from spleens of five of 29 Calomys rodents trapped in the area. The Department of the Beni is divided into four interfluvial plains by the principal rivers which run north and south. The presence of Machupo virus had been found in three of these plains during the years 1963-1969. Although the Beni is an underdeveloped area, progress in transportation and communication is beginning. The appearance of Machupo virus in 1971 west of the Rio Mamore, in the fourth and largest interfluvial plain causes one to speculate whether progress in transportation will result in progression of infected rodents and human disease.

A long-term, follow-up study of former San Joaquin inhabitants was made in November 1971. On the basis of data obtained in 1963, four categories of patients were selected: 1) clinical case-serologic specimens not available; 2) clinical case-serologically confirmed; 3) clinical case-serologically negative; and 4) persons with no history of clinically compatible illness.

Prompted by the finding of a mediastinal lymphosarcoma in one of the principal investigators of this project, approximately 380 persons were interviewed, and examined physically; serum, blood smears, and chest X-rays also were obtained. All identifiable deaths since 1963 were recorded together with the presumptive reasons for death as given by family members. No tumors were found, but good cohorts of patients and controls were established and at least 50 sera had a Machupo neutralizing antibody titer of 1:64 or greater. This is of considerable interest in view of the plan of the Bolivian Ministry of Health to collect large volumes of sera with high titering neutralizing antibody for gamma globulin processing to be used in early treatment of disease or in prevention of contact infections.

Biology of Tacaribe Viruses: A virus strain recovered from human spleen was passaged 110 times in specific pathogen-free suckling mice. After 20 passages this strain no longer induced viremia in marmosets or death in guinea pigs. Beyond 50 passages it would no longer multiply in human lymphoblasts. At the 80th passage, no growth was observed in human diploid cells and plaque characteristics in Vero cells had changed. We are attempting to select virus clones non-virulent for animals but capable of growth in diploid human cells. Concentration and inactivation of such virus should produce a candidate vaccine in the near future.

The project to genetically select subcolonies of immunocompetent and immunotolerant Calomys callosus has been terminated. All animals have been inoculated with Machupo virus and analysis of the data is underway. Continued brother-sister mating and parent-sibling backcrosses finally resulted in decreased fecundity and increased cannibalization to the point where sublines could not be

propagated.

A study of the behavior of Machupo virus and Latino virus (non-pathogenic for humans) in colonies of Calomys from the San Joaquin area and from the Latino area is in progress. Both viruses also are being studied in hybrid Calomys; i.e., offspring of San Joaquin females mated with Latino males and vice versa. Preliminary results reveal that Machupo virus induced anemia, large spleens, viremia, and no circulating N antibody in colonized Calomys of both areas, whereas Latino virus does not induce anemia or change in spleen size. Challenge of Latino-infected Calomys with Machupo virus is planned. This work had been hampered by inability to develop a reliable plaque neutralization system for Latino virus, a problem recently resolved by use of liver-spleen homogenates from suckling hamsters infected by combined intraperitoneal and intracerebral inoculation with a virus strain previously plaque purified in cell cultures.

Miscellaneous Findings: The incidence of spontaneous tumors appearing in non-infected, colonized Calomys callosus was investigated during a two-year period. Twenty-five animals of each sex, approximately 10 months of age, were kept in separate cages and observed intermittently for tumors. At the end of the two-year period, there were seven male and three female survivors. The tumor incidence was 40% in males, and 28% in females. With the exception of a testicular tumour, and one cholangio-cellular carcinoma, all tumors were sarcomata.

A cell line has been established from a fibrosarcoma of the leg muscle of one Calomys callosus. The cells are predominantly fibroblastic in appearance, and it has not yet been determined if these are from the tumor or from the muscle tissue.

Techniques for growth and maintenance of primary Calomys embryo cell cultures have been established in preparation for work with Machupo virus designed to test whether the virus can produce malignant transformation or new virus-specified, host-specific antigens. Dr. John Coe, RML, has succeeded in purifying various immunoglobulins of Calomys callosus and is preparing a specific fluorescein-labelled reagent for study of responses and antibody localization in rodents infected with Machupo virus.

Significance to Bio-Medical Research and Future Course of Project:

Work on the biology of Machupo virus and vaccine preparation will be continued for another year under a contract. This should provide the basis for eventual successful control of hemorrhagic fever in Bolivia by a new research team. It is hoped that some way can be found to permit annual follow-up on health status of former BHF patients since this cohort represents the largest known groups of humans with a defined history of infection by any arenavirus.

Publications:

Peters, C. J., Webb, P. A., and Johnson, K. M.: Measurement of antibodies to Machupo virus by the indirect fluorescent antibody technique. Proc. Soc. Exp. Biol. Med., 1972 (in press)

Serial No. NIAID-100-C
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Arboviruses

Previous Serial Number: Same

Principal Investigators: Dr. P. H. Peralta and Dr. T. E. Walton

Other Investigators: Dr. K. M. Johnson, Dr. R. B. Tesh, Lt. Col. G. A. Eddy, Dr. D. H. Martin, and Miss G. Oro

Cooperating Units: MAG-FAO: Dr. R. Horrell, Ing. O. Miranda
Veterinary Laboratory, Ministry of Agriculture, Panamá: Dr. O. Alvarez
Gorgas Memorial Laboratory: Dr. P. Galindo, Dr. A. Adames, and Dr. M. Grayson

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	8 2/12			8 2/12
Professional:	4			4
Other:	4 2/12			4 2/12

Project Description:

Objectives:

To develop methods for measuring arboviruses and their antibodies; to study in detail the pathogenesis, epidemiology, and ecology of various arthropod-borne virus infections in man and animals; and to define the antigenic relationship of members of selected serologic groups or complexes within the arbovirus class.

Methods Employed:

Standard field and laboratory procedures are used for isolation of viruses and detection of antibodies in man and a wide variety of animals. A longitudinal study approach is applied in certain instances to the study of

various natural populations.

Major Findings:

Venezuelan Equine Encephalitis (VEE).

Epidemiology. Serological evidence obtained in 1971 from canine, porcine, and equine populations in the southern Pacific region of Nicaragua indicated that equine-virulent VEE virus continued to be present there after an equine epizootic had been suppressed by vaccination, and that the seacoast ecology might be important in its maintenance. Isolation of 12 VEE virus strains from the crabhole mosquito Deinocerites pseudes (D&K) during the 1970 epizootic in the adjoining Costa Rican province of Guanacaste suggested that this insect, whose habitat is restricted to the seacoast, might be an important link in the as yet undemonstrated endemic cycle of virulent VEE virus. In August, 1971, mosquitoes were trapped in Nicaraguan Pacific coastal estuaries in an attempt to document the continued presence of virus in the area; 38,881 mosquitoes were captured by light trap, 99% of which were D. pseudes. No isolations were made. Blood-engorged mosquitoes were not assayed for virus but were tested to determine source of blood. Of 242 blood meals examined, 79% fed on mammals, 10% on birds, and 6% on reptiles or amphibians. Of the mammalian feedings, 58% were on bovines, 13% on equines, and only 7% fed on rodents or other mammal orders. VEE endemic cycles presently known depend upon rapid turn-over of rodent populations for virus maintenance. Feeding patterns of the Nicaraguan D. pseudes study population do not involve rodents to a significant degree, suggesting that establishment of scuh a cycle by this mosquito is unlikely. Feedings on birds, however, particularly herons which are known to experience viremia sufficient for cyclic transmission to mosquitoes, indicates need for further investigation of this ecologic association.

Experimental Equine Infection. Since last year's annual report, we have continued our horse inoculation studies utilizing strains of VEE virus. Six horses, vaccinated 14 months previously with the strain TC-83 attenuated VEE vaccine, were challenged using equine-virulent VEE virus (strain MF-8, subtype I, variant B) from the 1969-1971 Central American epizootic. All animals resisted challenge; no viremia, fever, leukopenia, or clinical illness was observed. Increases in neutralizing antibody titers to all of six heterotypic VEE virus strains tested were recorded; titers against the vaccine virus and its parent strain, Trinidad donkey #1, did not attain levels seen at a similar time after initial vaccination, while those to the other four groups exceeded the initial response.

A group of four animals vaccinated with the equine-avirulent Mena III strain (subtype I, variant E) 15 months previously were also challenged with subtype I-B virus. Responses were essentially similar to those for TC-83 vaccinated horses, demonstrating that Mena II virus acts as a natural VEE vaccine for equines in areas where it is endemically active such as Panama and parts of Central America.

A group of four horses was inoculated for mosquito infection trials utilizing subtype I-B, VEE virus and three species of mosquitoes, Deinocerites pseudes, Culex (Melanoconion) aikenii, and Mansonia titillans, with the cooperation of the Gorgas Memorial Laboratory. Mosquito exposure to these animals was restricted to periods when expected viremia was in the 2-4 dex range/ml of blood. Only two D. pseudes were infected, and the highest viremia was about 3.5 dex/ml. suggesting that these species are not more sensitive to VEE virus by ingestion than several others recently studied in the United States.

Two groups of four horses each were infected with a high dose ($10^{7.8}$SMCLD$_{50}$) and low dose ($10^{3.8}$SMICLD$_{50}$) of variant B, subtype I (strain MF-8) of VEE virus to determine differences, if any, of clinical and virological response. All four horses given large amounts of VEE virus were clinically ill, circulated high concentrations of virus in the blood, and developed CF antibodies. One animal died. Two horses infected with small amounts of virus exhibited severe clinical illness, and had high viremia levels whereas the other two horses were mildly ill and not detectably viremic. All four horses developed CF antibodies. Cerebrospinal fluid obtained by cisternal puncture contained virus when animals were viremic. Virus-positive fluids also contained greatly increased amounts of protein, but very few cells, suggesting that vascular "leaks" were produced in viremic horses.

Antibody profiles have been constructed for all horses infected experimentally with epizootic and enzootic VEE virus strains. Using the neutralization, CF, and HAI tests, antibody titers to many of the VEE variants of antigenic subtype I have been determined for varying periods of time. The data demonstrate basic antigenic differences between these two classes of viruses, in that quantitative antibody responses are generally higher to the homologous agent and its relatives of similar equine virulence. Equine virulent strains produced more antibody, no matter how measured.

Virus Characterization: Plaque morphology and pH optimum for hemagglutinins were investigated further as phenotypic markers that correlate with VEE virulence for equines. Standardized tests were carried out with 116 virus strains. Small plaque size and a sharp pH optimum for hemagglutinins were clearly related to equine virulence of viruses as determined both epidemiologically and experimentally. Plaque size also was related to antigenic properties of viruses of the VEE complex. It was found that small plaque size in Vero cells was the result of sensitivity of these strains to polyanions such as sulfated polysaccarides present in agar.

Additional work was directed to the search for genetic differences among several strains of VEE virus. A series of experiments was carried out using three known horse-virulent strains, three known avirulent strains, and two unknown but probable avirulent strains. Properties tested included temperature sensitivity, pH absorption characteristics, UV sensitivity, DEAE cellulose column chromatography, differential characterization by chromium$_{51}$ release from infected labeled cells, growth curve characteristics, growth in different cell lines, aggregation and disaggregation of magnesium chloride, and time-to-death

studies in adult mice. With the possible exception of the time-to-death studies, none of the others showed sufficient distinction between strains to warrant their use for such purposes.

Immunofluorescent studies of affected horse brains are in progress which show specific indirect fluorescence. This is particularly noted along axons and axon sheaths. Elution of brain sections with low pH buffer to remove interfering antibody increases the intensity of the fluorescent reaction.

Phlebotomus Fever Group. This group of agents has been poorly studied, thus in order to identify our sandfly viral isolates it has been necessary to examine the serologic relationships of the known members of the group. Cross-neutralization and complement fixation tests were performed on the 12 known members of the Phlebotomus Fever Group as well as several of our sandfly isolates. With the exception of Icoraci virus, hyperimmune serum prepared against each of the 12 agents was specific by both neutralization and complement fixation tests. This is in contrast to results obtained by hemagglutination inhibition tests which demonstrate cross-reactivity between various members of the group. Complement fixation tests were then carried out on all of our sandfly virus isolates. Preliminary studies indicate that in addition to VSV-Indiana, Changuinola, and Chagres virus, we have several new viral agents. Prototypes of the new agents have been sent to the Yale Arbovirus Unit, New Haven, Conn. for confirmation and identification.

Significance to Bio-Medical Research and the Program of the Institute:

This year's results extend and confirm the fundamental relationships previously noted between several characters of VEE virus strains and their natural behavior. These markers are of immediate usefulness in the continuing search for understanding of the origin and maintenance of equine-virulent and -avirulent viruses. The field work in Nicaragua, though virologically negative, suggests that further study of specific kinds of birds might prove rewarding in the search for an inter-epidemic virus cycle of equine-virulent virus.

The new data from experimentally infected horses demonstrates clearly that this animal and the various virus strains provide a highly promising model system for the elucidation of the pathophysiology of encephalitis. Viremia is clearly correlated with, and precedes disease of the central nervous system. Onset of encephalitis is temporally correlated with appearance of detectable circulating antibodies. Appearance of virus and protein in the cerebrospinal fluid indicates a vascular lesion which could be produced either by direct viral damage to endothelial cells or as a consequence of formation of antigen-antibody complexes.

Characterization of Phlebotomus fever virus serotypes occurring in Panamá will permit rapid assessment of their potential for causing human disease, using serums from animals and humans available in our serum bank that contains more than 100,000 catalogued specimens.

Proposed Course of the Project:

Work on all aspects of these problems will be continued for another year under a contract.

Honors and Awards:

Dr. T. E. Walton

Invited to present a paper entitled "Encefalomieltis equina de Venezuela experimental en equinos." IIL Congreso Centroamericano, I. Nacional de Microbiologia. Guatemala City, Guatemala, July, 1971.

Invited participant at PAHO Workshop-Symposium on VEE. Washington, D.C., September, 1971. Presented two papers entitled "Field and experimental evaluation of the attenuated VEE vaccine, strain TC-83" and "Virulence properties of VEE virus subtypes in horses." These papers will be published in a PAHO monograph.

Invited participant at a USDA Workshop on VEE. Beltsville, Maryland, March, 1972.

Lt. Col. G. A. Eddy

Invited to present paper entitled "An overall view of Venezuelan equine encephalomyelitis in the Americas" at International Round-Table on Venezuelan Equine Encephalomyelitis, Mexico City, Mexico. May, 1971.

Invited participant at Pan American Health Organization Workshop-Symposium on Venezuelan Encephalitis Virus, Washington, D.C., September, 1971. Read paper entitled "Equine encephalomyelitis in Costa Rica, 1970."

Invited participant, and president of session on vaccines, at First National Seminar on Venezuelan Equine Encephalomyelitis, sponsored by Colombian Ministry of Public Health and the Pan American Health Organization. Bogotá, Colombia, November 1971.

Dr. D. H. Martin

Invited to present a paper on "Epidemiologica de la encefalomielitis equina de Venezuela. Resultados de estudios de 1970 en Costa Rica" at III Congreso Centroamericano, I Nacional de Microbiologia. Guatemala City, Guatemala, July, 1971.

Invited participant at Pan American Health Organization Workshop-Symposium on Venezuelan Encephalitis Virus, Washington, D.C., September, 1871. Discussant of paper "Serologic differentiation of viruses of the VEE Complex."

Dr. K. M. Johnson

Invited participant, and Chairman of session on Host-Parasite Interaction at Pan American Health Organization Workshop-Symposium on Venezuelan Encepalitis Virus, Washington, D.C., September 1971.

Publications:

Eddy, G.A., Martin, D.H., Reeves, W.C., and Johnson, K.M.: Field studies of an attenuated Venezuelan equine encephalomyelitis vaccine (Strain TC-83). Infection and Immunity., 5: 160-163, 1972.

Martin, D.H., Eddy, G.A., Sudia, W.D., Reeves, W.C., Newhouse, V.F., and Johnson, K.M.: An epidemiologic study of Venezuelan equine encephalomyelitis in Costa Rica, 1970. Amer. J. Epidemiol., 1972 (in press).

Walton, T.E., and Johnson, K.M.: Experimental Venezuelan equine encephalomyelitis virus infection of the bovine. Infection and Immunity., 5: 155-159, 1972.

Walton, T.E., Brautigam, F.E., Ferrer, J.A., and Johnson, K.M.: Epizootic Venezuelan equine encephalomyelitis in Central America: Disease pattern and vaccine evaluation in Nicaragua, 1969-1970. Amer. J. Epidem., 95: 247-254, 1972.

Walton, T.E., Alvarez, Jr., O., Buckwalter, R.M., and Johnson, K.M.: Experimental infection of horses with an attenuated Venezuelan equine encephalomyelitis vaccine (Strain TC-83). Infection and Immunity., 1972 (in press).

Serial No. NIAID-100-D
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Korean Hemorrhagic Fever

Previous Serial Number: Same

Principal Investigator: Dr. N. H. Wiebenga

Other Investigator: Dr. K. M. Johnson

Cooperating Units: None

Man Years	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To study the immunoglobulin response in patients presenting the syndrome of Korean hemorrhagic fever (KHF) and to explore the relationship of KHF antibody to some of the viral antigens of the Tacaribe group viruses.

Methods Employed:

About 24 patients were selected from whom three or more serial serum samples taken at weekly intervals were available for testing. These sera were tested in commercially supplied radial diffusion plates for IgM, IgG, and IgA. Selected sera were also tested by the indirect fluorescent antibody test (IFAT) using Vero cell cultures infected with Latino virus as viral antigen.

Major Findings:

In all of the patients sera tested there was an increase above normal values in IgM, while the values for IgG and IgA remained within normal limits. In general, the amount of IgM fitted a curve of increasing values during the first week after admission to the hospital and of decreasing amounts during the second and third weeks of hospitalization. There was no apparent change in the

values of IgA and IgG during similar periods of hospitalization.

It is important to note that all patients studied were admitted from four to seven days after their onset of historical, clinical complaints. In every case, the initial serum sample obtained on the day of admission contained quantities of IgM in excess of normal values.

When these sera with high IgM values were tested by IFAT against Latino-virus-infected Vero cells, some demonstrated fluorescence that was not apparent in uninfected cell controls. However, the degree of fluorescence and the reliability of obtaining fluorescence in Latino-virus-infected cells with positive control sera precludes any firm conclusion.

Control and reproducibility of Latinovirus infections in cell cultures remains a problem that we do not understand.

Significance to Bio-Medical Research and the Program of the Institute:

The finding of higher than normal IgM values in the admission sera of patients with KHF (from four to seven days after onset of symptoms) may be significant in planning future studies concerned with isolating the etiologic agent of infection (presumably viral). If this increased amount of IgM represents specific antibody against KHF "virus," virus isolation studies on patients admitted to hospital must be doomed to failure since they seem to be immunized by the time of their admission.

The lack of any secondary infections among hospital personnel caring for KHF patients during the past 20 years is an interesting epidemiological note. This evidence has been cited to suggest that person-to-person spread does not occur, in contrast to other hemorrhagic fevers. If most (or all) patients are non-infectious due to antibody formation before admission to hospital, the possibility of person-to-person spread must be re-evaluated.

Proposed Course of the Project:

The priorities of Health Care and Research in the United States no longer apply to this study. It has been discontinued until another sponsor can be found.

Serial No. NIAID-101
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Hepatitis and Australia Antigen in Panamá

Previous Serial Number: Same

Principal Investigators: Dr. C. J. Peters and Dr. W. C. Reeves

Other Investigators: Dr. K. M. Johnson, Mr. J. Vogel, and Mr. Ross Buckwalter

Cooperating Units:
Ministry of Health, Republic of Panamá
Hospital del Seguro Social, Republic of Panamá
Hospital del Niño, Republic of Panamá
Hospital Santo Tomás, Republic of Panamá
Gorgas Hospital, Canal Zone
Office of the Chief Surgeon, United States Army, Southern Command
Dr. Carmelo Ciniglio; University of Panamá
Laboratory of Viral Diseases, NIAID, NIH; Dr. Robert H. Purcell and Dr. Jerrold J. Lander; Project Serial No. NIAID-70-D.
Dr. Paul Holland, Clinical Center Blood Bank, NIAID NIH
Dr. Normando Arbelaez, Universidad Nacional, Bogotá, Colombia
Dr. Efrain Solarte Alve, Chief of Leprosy Control, Ministry of Public Health, Bogotá, Colombia
Dr. Agusta Takeda, Instituto Adolfo Lutz, São Paulo, Brazil
Dr. C. Eduardo Rocha Telles Rudge, Divisão de Hansenologia e Dermatologia Sanitaria, Secretariat of Health, São Paulo, Brazil.

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	4 6/12			4 6/12
Professional:	2 8/12			2 8/12
Other:	1 10/12			1 10/12

Project Description:

Objectives:

1. To investigate the epidemiology of Au antigen and acute hepatitis in Panamá.

2. To study the genetics and epidemiology of the carrier state for Au antigen in a detailed longitudinal study among the indigenous peoples of Panamá.

3. To inoculate certain Panamanian primates with Au antigen, based on their availability and relevance for the epidemiology of Au antigen in Panamá.

4. To evaluate the relevance of serum immunoglobulin levels to the study of hepatitis.

5. To evaluate the relation of certain chronic infections to infections by cytomegalovirus (CMV), Epstein-Barr (EB) viruses, and the presumed agent of one type of hepatitis as measured by Au-antigen and -antibody techniques.

Methods Employed:

Au antigen was determined by gel diffusion (GD), complement fixation (CF), and immunoelectrophoresis (IEOP). Immunoglobulin concentrations were determined by radial immunodiffusion using commercial antibody-in-agar plates. CMV and EB antibodies were determined by the CF and indirect fluorescent antibody tests (IFAT) respectively. Antibodies to Au antigen were measured by radioimmunoprecipitation (RIP) in the LVD, by Drs. Purcell and Lander.

Major Findings:

Hepatitis and Au infection among non-indigenes: Longitudinal studies of family contacts of urban, acute, Au-positive hepatitis patients revealed that about 10% (5/61) were secondarily infected. Four of the five persons infected had no identifiable illness, and the fifth had icteric hepatitis. Fifty-two contacts of Au-negative hepatitis patients failed to show evidence of infection These studies (done by Drs. Lander and Purcell, LVD) demonstrate intra-familial spread of Au infection in a natural population and show that anicteric infectio is a frequent occurrence.

Population studies of the carrier state revealed a non-Indian community (El Aguacate) in the interior of Panamá which has a significantly higher frequency of Au than other groups in the country. El Aguacate had 9/187 (4.8%) positive as compared to 5/1186 (0.4%) for the rest of the towns in the interior Studies are planned to elucidate possible causes for this difference. Sera from Costa Rica, a country which has been reported to have high rates of Au-positive hepatitis, were tested for Au but there was no significant difference in the carrier rate when compared to Panama (C.R., 4/571 = 0.7%; Panama,

16/2008 = 0.8%). Prostitutes and nuns in Panama, and diabetics from Gorgas Hospital, were screened and not found to differ strikingly from the general population. Pilot studies were carried out with Colombian and Brazilian lepers. Lepromatous lepers had an increased incidence of antigenemia, but it has not been possible to obtain comparable groups in terms of duration of hospitalization, treatment, et cetera. Dr. Normando Arbelaez, visiting in our laboratory, has shown that prevalence of Au in a Colombian population of low socio-economic status and poor sanitation is very low.

The value of IgM testing is being evaluated in a larger group of patients. Initial IgM values from a total of 484 clinically and biochemically confirmed acute hepatitis patients (116 Au positive and 368 Au negative) were used to construct a histogram of the distribution. The Au positives had a modal IgM of about 2.5 mg/ml confirming our previous data. With the larger number of Au negatives in this sample, we were able to show a bimodal distribution with modal values of 5.5 and 1.5. The Au-negative cases with low IgM comprise about 15% of this group.

The clinical picture of Au-positive hepatitis was compared with that of Au-negative hepatitis, using adult cases only. A questionnaire was administered to 35 Au-positive and 50 Au-negative patients. The Au-positive group had a more gradual onset, a longer prodrome, less fever, less vomiting, more frequent arthralgia (7/35 versus 0/50) than Au-negative patients. Study of charts from two hospitals revealed that the Au-positive cases were more severe as measured by maximum bilirubin, maximum SGOT, and duration of hospitalization.

Au infection and chronic antigenemia among indigenes: The prevalence of Au and anti-Au antibody have been determined in representative samples of each of Panamá's major Indian groups. Significant differences occurred by Indian tribe, both in the number of people exposed to Au and in the proportion of exposed individuals who became chronically antigenemic. Among the Guaymi Indians of the Pacific coastal mountains 23 of 806 tested (3%) were antigenemic, whereas among Guaymi of the Atlantic coastal region 20/267 or 8% were antigenemic. The prevalence of antigenemia was significantly lower in other Indian tribes; of 298 Chocó tested, only 2 (0.7%) were antigenemic and none of 273 Cuna Indians had Au. Among both Guaymi groups and the Chocó, exposure to Au, as indicated by anti-Au antibody, was relatively high (Pacific Guaymi 56/181, Atlantic Guaymi 87/216, Chocó 93/222) as compared to the Cuna (12/122).

Au antigenemia appeared to have a familial pattern of occurrence among the Pacific Guaymi. Besides detailed family geneologies, we have collected serum specimens, glycerolated red cells, and red cell hemolysates from approximately 500 individuals representing the families of antigenemic Indians and matched control families. We are currently confirming geneologies by typing appropriate specimens as to haptoglobin, Rh system, phosphoglucomutase and lactic dehydrogenase. Because of the complexity of the data and the necessity of comparing multiple variables between families, computer programs are being devised to further analyze this information.

To date, 300 Guaymi Indian sera have been tested for antibodies to EBV. There was a progressive increase in the percentage positive with age, with 10% positive at five years and more than 50% positive at 40 years and above. We also tested 200 sera from an urban Panamanian community for anti-EBV antibody, and in contrast to the Indian data a large number of positives (40%) occurred in all age groups. In an attempt to evaluate possible relationships of this chronically infectious agent to chronic antigenemia, we are devising computer analysis programs to compare frequencies and titers between populations and also between families.

In conjunction with Drs. Robert Purcell and Paul Holland, LID, 330 Au-positive sera, representing all positive individuals from Panamá's Indian community, acute hepatitis patients from Panamá City, chronically infectious carriers from both rural and urban Panamá, and lepromatous lepers from Colombia and Brazil, are being tested for Au subtype. This is being done because of the possibility that different subtypes of Au may exist in different geographic areas, and that chronic antigenemia may be caused by specific subtypes.

Significance to Bio-Medical Research and the Program of the Institute:

It is likely that increasing medical administration of blood products and illicit parenteral narcotics use will place all aspects of hepatitis research into a position of high priority. Elucidating modes of transmission, other than parenteral, of Au-positive hepatitis has obvious practical importance. Similarly, exploration of the possible mechanisms accounting for existence of a chronic, asymptomatic, carrier state has enormous significance. In the Republic of Panamá, three genetically distinct, free-living, indigenous populations, as well as racially mixed urban and rural populations, live in relatively close geographic proximity but under a variety of epidemiologic situations. This allows a unique opportunity to explore the epidemiology of Au in nature.

Proposed Course of the Project:

Because of the quantity of data involved in analyzing the acute hepatitis patients and dealing with the Indian families, we are developing computer methods to deal with clinical, epidemiologic, laboratory, and urologic tests in individuals and to deal with family patterns. The family analysis, in particular, will be applied to antibodies to Au, Epstein-Barr virus, and cytomegalovirus.

The study of Au antigenemia among the Indian groups will be continued. Rh antigens, phosphoglucomutase and haptoglobin typing will be used to evaluate paternity in critical families and to assess genetic background of closely related groups with differing frequencies of antigenemia. Sera will be obtained from Cuna Indians living in the San Blas Islands, to confirm the low frequency of anti-Au in this group. Cuna who live in the Darien under conditions similar to the Chocó Indians also will be sampled. Clues to the possible route of transmission of Au in these groups will be sought by comparing frequency of

antibody to agents transmitted by different routes to frequency of anti-Au.

The Au-negative, acute hepatitis patients who did not develop an IgM elevation will be studied further. Convalescent sera will be tested for anti Au, and serologic studies for other agents will be carried out. They will be tested for Au antigen by immunoassay if this can be arranged on a collaborative basis.

Once the appropriate data-processing tools are available, multivariate approaches to analysis of the clinical features and epidemiologic patterns of Au-positive versus Au-negative hepatitis will be completed.

Contacts with Colombian and Brazilian leprosaria have produced preliminary serum samples which show increased frequency of Au in institutionalized lepromatous lepers, but these collections must be enclarged to include non-institutionalized lepers.

Investigators from Costa Rica and Chile will be trained at MARU to do Au-antigen testing. This is an important part of the overall effort to understand the biological behavior and mode of transmission of Au by developing capability for Au testing using standard methods in many countries.

Publications:

Peters, C. J., and Johnson, K. M.: Serum immunoglobulin levels in Au-antigen positive and Au-antigen negative hepatitis. Clin. Exp. Immun., 1972 (in press)

Honors and Awards:

Dr. C. J. Peters:

Invited to present a paper on "Australia antigen: Studies in Panamá and implications for the control of hepatitis" at the XIV Congreso Médico Centroaméricano, held at the University of Panamá, December, 1971.

Serial No. NIAID-102
1. Middle America Research Unit
2. Virus Diseases Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Nutritional, Immunological Surveys of Human Populations in Central America and Panamá

Previous Serial Number: Same

Principal Investigator: Dr. K. M. Johnson

Other Investigators: MARU: Dr. P. H. Peralta and Mr. R. M. Buckwalter
INCAP: Dr. L. J. Mata

Cooperating Units: Institucion de Nutricion de Centro America y Panamá, Guatemala
Pan American Health Organization; Pan American Sanitary Bureau; Zone III Office, Guatemala
National Governments of Costa Rica, El Salvador, Guatemala, Honduras, Nicaragua, and Panamá

Man Years:	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	3			3
Professional:	1 4/12			1 4/12
Other:	1 8/12			1 8/12

Project Description:

Objectives:

To determine prevalence of specific antibodies to a large number of arbo- and other viruses and to certain parasites among families carefully selected on a random basis in each of six countries. These data will then be related to detailed information being collected on nutritional, socioeconomic parameters of these individuals by INCAP and cooperating organizations.

Methods Employed:

Family groups are selected statistically well in advance of actual work. Serum samples are obtained in the towns and villages at the time of large scale clinical and biochemical surveys. Sera are divided with aliquots stored at INCAP and MARU for testing by standard techniques for virus and

parasitic antibodies. Complement-fixation testing with exo-antigen from culture forms of Trypanosoma cruzi is used for Chagas' disease testing and the indirect fluorescent antibody test is used for toxoplasmosis. Hemagglutination-inhibition and cell culture plaque neutralization tests are employed for arboviruses. All field data and serum inventories are handled on IBM cards.'

Major Findings:

Group B Arbovirus Antibody Survey. This year the testing of children's sera from appropriate communities was completed and testing of selected adult sera against dengue serotypes 1, 3, and 4 and/or Bussuquara virus approached completion. Major findings that have emerged from the combined work of the past several years may be summarized as follows.

Testing of more than 5,000 adult sera from the INCAP-ICNND nutrition survey of 1965-67 in Central America and Panama by hemagglutination inhibition (HI) provided little evidence of group B antibodies in the highlands of Guatemala and Costa Rica, but revealed much HI reactivity, frequently broad in pattern, in sera from other areas. With the HI results as a guide, a large subsample of these sera was selected and tested for antibodies by a plaque neutralization (N) test using Vero cell microcultures in multiwelled disposable trays, in order to interpret the HI findings and to obtain an estimate of human antibody prevalence to various group B arboviruses in the region.

Preliminary N tests indicated that Powassan and Bussuquara antibodies were rare, but recent screening of sera from Panama (the country with the greatest group B, HI-positivity) have shown that 4.7% of 682 adults tested had N antibody to Bussuquara, a virus which has been isolated in Panama in the past.

All sera from adults (≥ 15 yr. old) from 123 of the 190 survey communities were examined in N test against yellow fever (YF), dengue 2 (strain TH-36), Ilheus and St. Louis encephalitis (SLE) viruses; and three other towns were tested against YF only. Included were a few of the towns which had been negative by HI (a finding confirmed by N test) and the majority of the HI-positive towns—the rest being omitted as they were located close to, and had HI patterns similar to, selected communities. In all, 3,536 adults (1,626 male, 1,910 female) were tested against all four viruses. Children's sera were tested against a given virus in those communities showing greatest antibody prevalence; 2,953 sera were tested against one or more of the agents.

It was found that in five countries (all except El Salvador) YF antibody prevalence among adults was relatively high (37% overall) with 53 of 105 communities showing ≥50% prevalence. There was no difference between the sexes. and prevalence was quite uniform among all age groups 20 years and over. This finding was compatible with vaccine-induced infection, and in fact these were the five countires in the path of the last epizootic wave of YF that moved from Panamá to Guatemala between 1948 and 1956, and where human vaccination campaigns were carried out. The finding of far less YF N antibody (6% over-all in adults

of El Salvador but 17% among males over 40) suggested that YF virus was active in that country prior to 1940.

Dengue 2 over-all antibody prevalence among adults was 15% in towns studied, there being no difference between the sexes. Children under 15 were without antibody, there were only scattered positives between ages 15 and 25, but prevalence increased thereafter until it attained about 30% by age 50. Only five positive persons were born after the last documented Central American dengue epidemic in 1942 (in Panamá). Data for "dengue towns" (≥ 15% prevalence) suggested that in the decades prior to eradication of Aedes aegypti, two-thirds of the residents were infected by age 35-40, and that now more than half of the present residents of these communities are susceptible to dengue 2 infection.

A group of more than 600 sera from towns in Nicaragua and Honduras was tested for antibodies to dengue type 1, and a smaller subsample against types 3 and 4. Another 150 sera from "dengue towns" in Panamá are currently being tested for N antibodies to these same three dengue serotypes. Data obtained thus far suggest that a dengue-2 infection produces dengue-1 neutralizing antibody two-thirds of the time. Dengue 1 may have been active in Central America, however, in the early decades of this century (the youngest of the few monovalent positives was born in 1908), while dengue 3 very likely was active in Panamá, and possibly in other countries, before A. aegypti eradication. No evidence for presence of dengue 4 was obtained

Ilheus and SLE viruses, in contrast to YF and dengue, are continuing to cause human infections in this region. These viruses appear to be neither very prevalent nor ubiquitous. The over-all adult prevalence was 10% for Ilheus and 6% for SLE. Both viruses have been more active in persons surveyed in Panamá than elsewhere. An over-all excess of antibody in males suggests that infections are acquired away from home.

Significance to Bio-Medical Research and the Program of the Institute:

Ecologic maps of the distribution of human infection with a variety of arboviruses and protozoa having clear-cut present or potential future public health importance in Central America and Panamá are emerging from this project. The survey has provided and is still providing clues as to the factors that govern the transmission of these various infections and as to the sites where we or other research groups may expect to be able to carry out profitable direct investigation of them.

Proposed Course of the Project:

Statistical analyses involving antibody patterns and a variety of ecologic parameters are to be carried out for the group B arboviruses as well as for Cache Valley virus (for which N testing was done previously). Manuscripts will be prepared for publication.

Testing of the same serum collection for antibodies to other viruses, for epidemiological purposes, in connection with other projects (e.g. sandfly-associated-viruses, from projects No. NIAID-100C, NIAID-104-B) is planned for the near future, even though the present project, NIAID-102, will be considered terminated once the reports on the work described above have been concluded.

Honors and Awards:

Dr. K. M. Johnson:

Member, PAHO Scientific Committee on Dengue. Presented paper entitled "Survey for antibodies to selected group B arboviruses in humans of Central America" at 2nd Committee Meeting, March 23, 1972.

Dr. P. H. Peralta:

Invited to present paper entitled "Hallazgos preliminares de una encuesta sobre niveles de inmunidad a ciertos arbovirus del grupo B en poblaciones de Centro-America y Panama, 1965-1967" by P.H. Peralta and K. M. Johnson, at the Arbovirus Roundtable of the Third Central American--First National Congress for Microbiology in Guatemala City, Guatemala, July 25- 30, 1971.

Serial No. NIAID-104-A
1. Middle America Research Unit
2. Parasitology Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of Parasitic Infections in Panama and Central America

Previous Serial Number: Same

Principal Investigators: Lt. Col. J. C. Burke, MSC
(Medical R&D Command, U.S. Army)

Other Investigators: Maj. L. Ackerman, VC, Maj. L. Rutledge, MSC, Capt. W. T. Collins, MSC, and Dr. W. H. Dietz, NIAID

Cooperating Units: Office of Chief Surgeon, USARSO
Military Dispensaries in the Canal Zone (Army, Navy, and Air Force)
Gorgas Hospital, Canal Zone

Man Years:

	USAMRU Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	8 9/12			8 9/12
Professional:	3			3
Other	5 9/12			5 9/12

Project Description:

Objectives:

1. To clarify routes of transmission, natural reservoir hosts, and pathogenesis of several endemic parasitic organisms.

2. To establish the extent and importance of several of the most significant parasitic infections endemic in this area.

3. To improve methods for diagnosing such infections in the laboratory.

Methods Employed:

The basic techniques are serological testing of human and other animal sera with species-specific antigens in the indirect fluorescent antibody test (IFAT), in conjunction with culturing, animal inoculation, vector dissection, sentinel animal monitoring and tissue examination to determine infections and host response to organisms causing cutaneous leishmaniasis. Giemsa-stained

thick and thin blood smears are examined microscopically for microfilaria, malaria, and trypanosomes.

Major Findings:

Leishmaniasis. Since the last report, this laboratory has diagnosed 30 human cases of primary cutaneous leishmaniasis in North American personnel residing in the Canal Zone. These cases, along with the 24 cases diagnosed early in 1971, represent a substantial increase over previous years of 1965-1970, which had a case identification rate of 5-1/2 year.

Most of these patients acquired the infection within the Empire Range area on the Pacific side of the Canal Zone. Of the 54 cases, 22 had multiple lesions with the neck, face and lower arms most frequently involved. Infections have occurred in all racially represented groups and in approximate numbers to the size of their group in this community.

When personal history could indicate a specific site of probable exposure, a field study employing various techniques was established at the jungle site. These involved: vector collection and laboratory examination for identification and infection, small animal capture and laboratory examination for serological activity and organ-tissue infection, and sentinel animal exposure.

In cooperation with the Environmental Health Division, USARSO, sandfly populations were surveyed at selected locations within the Canal Zone (table 1). This survey indicates that L. panamensis and L. gomezi are the most widespread and abundant of the anthrophilic species present in the Canal Zone. Zoophilic species predominate in certain areas (Ft. Clayton, Empire Range) where transmission of Leishmania to military personnel is suspected.

Table 1. Sandflies identified by USAMRU from light-trap and CO_2 light-trap collection made by USAMRU and Environmental Health Division, USARSO over the period 1 July 1971 to 31 March 1972

Location	No. Identified	No. of Species	Prominent Species
Frijoles	83	3	L. panamensis
Fort Kobbe	1188	11	L. panamensis, L. gomezi
Howard Air Force Base	223	7	L. panamensis, L. gomezi
Farfan Naval Station	44	7	L. panamensis, L. gomezi, L. dysponeta
Empire Range	930	17	L. carpenteri, L. triramula
Rodman Naval Station	38	6	L. panamensis, L. carpenteri
Fort Clayton	112	5	L. carpenteri
Camp Chagres	13	6	
Limbo Field Station	488	19	L. carpenteri, L. aclydifera
Totals	3119	24	

Beginning in 1972, live collections from the Limbo Field Station and from the Empire Range have been dissected and examined for the presence of

flagellated organisms (Table 2). One specimen of L. shannoni collected on 17 March at the Limbo Field Station was found positive. The organism has been cultured in NNN medium and has been inoculated into Sigmodon for observation of Leishmania development. Other living collections from Fort Clayton and the Empire Range were inoculated into cotton rats without prior dissection in attempts to isolate Leishmania. These experiments have not yet produced positive results.

Table 2. Female sandflies dissected by USAMRU from collections made by USAMRU and Environmental Health Division, USARSO, over the period 1 January to 31 March 1972

	Limbo Field Station			Empire Range		
	January	February	March	January	February	March
L. carpenteri	52	9	67	0	2	100
L. triramula	2	0	1	0	3	120
L. camposi	4	1	5	0	0	24
L. aclydifera	11	5	12	0	0	0
Minor species	20	12	18	0	1	22
	89	27	103	0	6	266
Totals	219			272		
	491					

Tissue from a Proechimys sp. and a Didelphis marsupialis trapped on Fort Clayton were histopathologically negative for leishmanial organisms despite the IFA 1:8 titer in the D. marsupialis.

All patients have been treated with pentavalent antimonials. In all cases, there has been a rapid improvement of the lesion area, but in 12 of the 54 cases recurrence of tissue destruction by the organism has taken place. The IFA test, although an excellent diagnostic procedure, continues to be reactive 1-1½ years after apparently successful treatment. Home permanent addresses are available for all these patients and long term follow-up serological examination will be attempted.

The cotton rat (Sigmodon hispidus) is this laboratory's animal model for leishmaniasis. Ulcerative lesions have developed consistently in these animals when infective inoculum is of low passage cultured leptomonads or biopsied tissue from the edge of an active dermal lesion. Baseline normal hematological values are being determined for the various ages and sexes of these animals from our colony.

Preliminary experiments have been conducted to evaluate the influence of cellular and humoral antibodies to Leishmania forms in the cotton rat. Sera, spleen and lymph node cells from rats not exposed, with active ulcerative ulcers, and with healed scorified areas were used as donors to groups of rats receiving infective organisms and these viable biologicals. In one such experiment, all but one animal inoculated with leptomonads and various combinations of the biologicals became infected. The time required for swelling and reaction to the infective organisms was essentially the same for all groups.

Animals receiving normal and recovered spleen-lymph node cells developed open ulcers to a greater extent than the other animals. Of the animals surviving the experiment, 90% were able to resolve the swelling-ulcer response in 39-51 days although the animals receiving immune serum took the shorter time. Further experiments to assess the cellular and humoral roles in this infection are required. With increased technical ability for standardization and better knowledge of normal values this laboratory should contribute to the evaluation of organism and host interaction.

We have initiated immunologic studies on patients with cutaneous leishmaniasis. All patients are skin tested with a battery of antigens (PPD, Schick, Candida, Histoplasmin) and all have been found to have positive reactions to antigens other than the leptomonad. A leukopenia has been observed in three of six patients and elevation of gamma globulins by serum electrophoresis was found in two of five patients. Assays for blast transformation of lymphocytes and the production of macrophage migration inhibition factor (MIF) have shown the absence of MIF in two patients and normal lymphocyte transformation in two other patients. Migration inhibition factor and transformation studies of new patients are still in progress.

Skin lesions from Leishmania inoculated cotton rats have been examined by light microscopy. Numerous histopathology stains have been evaluated to determine which stain would best demonstrate the organisms in tissue sections. Thus far, hematoxylin without a counter stain remains to be the best stain. Future experiments are being planned to evaluate the histochemical reactions of L. braziliensis in an attempt to find a specific stain which will allow for rapid and accurate diagnosis of cutaneous leishmaniasis. Techniques utilizing fluorescent antibody staining still remain to be perfected to eliminate the non-specific staining of Sigmodon skin sections.

Significance to Bio-Medical Research and the Program of the Institute:

Complete agreement between indirect and direct diagnostic techniques for cutaneous leishmaniasis has been obtained in patients recently entering an endemic area and becoming infected. The indirect fluorescent antibody test is a reliable and rapid procedure to evaluate the presence and prevalence of Panamanian cutaneous leishmaniasis. Open cutaneous reactions of Sigmodon hispidus to leishmanial inoculations provides a laboratory model for further evaluation of the biological and immunological interplay of host and this parasitic protozoan.

Proposed Course of the Project:

To monitor the curative effect of pentavalent antimonials on cutaneous leishmaniasis and to evaluate the ability of indirect laboratory procedures. To assess the effect of treatment on individual patients. Attempt to improve the differential staining of leishmanial organisms and host tissue for rapid diagnosis and documentation of sequential dermal pathology. To test all new patients with fractionated leptomonad antigens by intradermal sensitivity, MIF and lymphocyte transformation to determine whether a differential response

occurs in the course of the disease.

Serial No. NIAID-104-B
1. Middle America Research Unit
2. Epidemiology Section
3. Panama Canal Zone

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Ecological Studies of Vesicular Stomatitis Virus

Previous Serial Number: Same

Principal Investigators: Dr. R. B. Tesh and Dr. B. N. Chaniotis

Other Investigators: Dr. K. M. Johnson, Dr. P. H. Peralta, Miss G. Oro, Maj. L. C. Rutledge, and Mr. R. M. Buckwalter

Cooperating Units: Ministry of Health, Republic of Panamá
Veterinary Section, Ministry of Agriculture, Republic of Panamá
Environmental Health Branch, U. S. Army Southern Command, Canal Zone
School of Public Health, University of California, Berkeley: Dr. W. C. Reeves
Yale Arbovirus Research Unit, Department of Epidemiology and Public Health, School of Medicine, Yale University: Dr. R. E. Shope

Man Years:

	NIAID Lab. Staff	Other NIH Personnel	Guest Workers	Total
Total:	6			6
Professional:	2 6/12			2 6/12
Other:	3 6/12			3 6/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
NIH-71-500CC	K.M. Johnson & L.D. Chance	Inter-American Foundation for Tropical Studies, Incorporated	E.L. Tyson	$5,500

Project Description:

Objectives:

1. To study the ecology of vesicular stomatitis virus (VSV) in an endemic area of the disease.

2. To study the biology of phlebotomine sandflies in both field and laboratory settings in order to better understand their role as vectors of VSV and other sandfly-borne agents.

3. To collect and process wild sandflies for the purpose of virus isolation, with subsequent identification and characterization of viruses obtained.

4. To establish laboratory colonies of local man-biting sandfly species in order to investigate their vector potential as well as the mechanism of experimental virus infection and transmission.

Methods Employed:

Standard virus isolation and serologic techniques were employed, using cell culture and animal inoculation. A new microplate precipitin test utilizing specific antisera produced in rabbits and chickens was used to identify blood meals of wild-caught sandflies. Insects used in virus transmission studies were housed in our new insectary. Regular field collections of wild animals and sandflies were carried out at two study sites in the Canal Zone and Panama under Contract NIH-71-500CC with the Inter-American Foundation for Tropical Studies. Results of serologic testing, animal trapping, and daily insect collections were summarized on IBM cards for subsequent tabulation and analysis.

Major Findings:

Viruses Isolated from Wild Sandflies. Regular collections of sandflies for virus isolation, blood meal identification, and laboratory studies were continued at two field stations located near Gamboa, Canal Zone, and El Aguacate, Republic of Panamá. A total of 235,975 wild-caught sandflies of both sexes were processed for virus isolation. Nine isolates of VSV-Indiana were obtained; three of the isolations were from pools of Lutzomyia trapidoi, a common anthropophilic species in Panamá. In addition to VSV, 237 other confirmed virus isolates were obtained, emphasizing the role of these tiny biting insects in the natural cycles of certain arboviruses. Interestingly, a number of the non-VSV isolates were made from pools of male sandflies. Since male sandflies are incapable of taking a blood meal, it indicates that they become infected by some other means than feeding on a viremic host and strongly implies transovarial transmission. These findings suggest that the epidemiology of sandfly-associated viruses may be quite different from the insect-vertebrate cycle of mosquito-borne viruses.

The 237 non-VSV isolates are presently under study. About 55% have been identified as Changuinola Group arboviruses; the remainder appear to be members of the Phlebotomus Fever Group. Of the latter group several isolates have been identified as Chagres virus (heretofore only isolated from sick humans); the rest appear to represent three or four new virus agents.

Experimental Infection and Transmission Studies. In view of our VSV isolations from naturally infected L. trapidoi, attempts were made to establish laboratory colonies of this species, and transmission studies were initiated. Wild-caught L. trapidoi were infected with VSV-Indiana by feeding on viremic infant hamsters. It was found that virus titers increased rapidly in infected female sandflies, and VSV transmission by bite was demonstrated as early as three days after the infective blood meal.

Because of repeated isolations of Phlebotomus Fever Group viruses from male sandflies by ourselves and other workers, and because of our inability to identify a susceptible vertebrate host which develops a sufficiently high viremia to infect a biting sandfly, the possibility of VSV-Indiana transovarial transmission was investigated. Four anthropophilic species (Lutzomyia trapidoi, L. ylephiletrix, L. sanguinaria and L. gomezi) were fed on viremic hamsters, and their offspring were reared and tested for presence of virus. Transovarial transmission of VSV-Indiana by L. trapidoi and L. ylephiletrix to about 20% of their offspring was demonstrated; both male and female progeny were infected. Virus was recovered from all developmental stages; mean virus titers from egg to first generation adult increased by 10^4, indicating that virus multiplication occurred during development of the sandflies. Virus titers in first generation adult females were comparable to those found in their parents. First generation infected female sandflies transmitted VSV-Indiana by bite to susceptible animals and in turn transmitted the virus transovarially to their progeny (second generation). These results demonstrate a possible mechanism for transmission and maintenance of this virus in nature without a vertebrate host reservoir.

Studies of Sandfly Biology.

Dispersal of phlebotomine sandflies in a tropical forest. Utilizing Helecon fluorescent powders (zinc sulfide), a mark-and-release program is currently in progress. The basic objective of this study is to determine both horizontal and vertical movements of sandflies with release of marked specimens at ground and canopy levels. Preliminary results indicate that sandflies move little as most recaptures of marked specimens were made within a 50-meter radius from the point of release. These findings are in marked contrast to those obtained with mosquitoes and may help explain the peculiar focal distribution of sandfly-transmitted diseases.

Studies of sandfly breeding sites. Sandfly breeding sites in the forest litter are being studied at the Limbo Field Station. Ecological data are acquired by emergence trapping and by supporting studies of the physical and biotic environment of the breeding populations. These data relate to species composition, density, prevalence and dispersion; to interspecies

relationships and community organization; to seasonal succession and population stability; to the population age-distribution; and to the physiographic, hydrologic, edaphic and vegetational conditions of sandfly breeding. The data are interpreted in terms of disease vector-potential and the methodology of vector control.

Current general results may be summarized as follows: Four major anthropophilic species breed in the forest litter, including the predominant man-biting species of the area under study (Lutzomyia trapidoi) and the predominant day-biting anthropophilic species (L. pessoana). L. trapidoi is the dominant species in breeding populations of the forest litter, accounting for nearly half of the species composition. This species breeds over some 80% of the forest floor with an average density of 9.8 per m^2. Populations are clumped, the greatest observed density being 48.5 per m^2. The remaining anthropophilic species (L. panamensis, L. gomezi and L. pessoana) occur in consistently smaller numbers, L. panamensis being more widespread, and L. pessoana being more concentrated. Sandfly species compositions and population densities in the forest litter appear to be largely determined by the patterns of distribution of the forest litter and currents of surface run-off, which are in turn determined by the physiographic and phytologic conditions prevailing in the study area. The control of these species in the immature stages of development does not seem feasible with soil treatment methods currently available. Control procedures directed at the adult stage are likely to remain the method of choice until the land is cleared and developed, at which time sandfly breeding may be expected to cease or to decline to manageable levels.

Studies of sugar feeding in phlebotomine sandflies. Hematophagous insects utilize sugars as a source of energy for their various activities and to sustain normal life span. Investigators are now becoming aware that the sugar feeding may be an important aspect in the development and subsequent transmission of parasites in hematophagous insects. The present study is the first step in determining some parameters related to the practical aspects of sugar feeding under experiment conditions. So far, results show that sandflies prefer high concentrations of fructose, sucrose, maltose, and glucose, in that order. Five of the nine sugars offered were unacceptable indicating a capacity of sandflies to discriminate.

Role of VSV-infected male sandflies in maintenance of virus in nature. Since our studies of transovarial transmission of VSV showed that male sandflies were infected as well as females, the question arose whether males are capable of infecting susceptible females during insemination. A project now in progress attempts to answer this question. The study has important epidemiological implications as we are seeking ways by which virus amplification might occur under natural conditions in the vector population.

Host-parasite relationship between VSV-Indiana and Lutzomyia trapidoi.

Two controlled experiments have been completed. VSV-infection had no effect

(adverse or beneficial) on the survival and fecundity of L. trapidoi when matched with non-infected controls reared under similar conditions. A third experiment with a different approach will be initiated in summer 1972.

Laboratory-rearing and colonization of L. trapidoi. After several failures in the past, we are now able to successfully rear L. trapidoi, allowing us to complete most of our experimental work related to VSV transmission. Colonization of this species requires considerable labor but we intend to proceed with it now that most of our time-consuming field studies have been completed.

Significance to Bio-Medical Research and the Program of the Institute:

Because of its economic importance and its resemblance to foot-and-mouth disease, vesicular stomatitis has long been of interest to veterinary and other animal health agencies. The disease occurs in sporadic epizootics in the United States, but is enzootic in many areas of Latin America. Until recently, the disease was thought to be limited to the New World, but recent isolations of a new VSV serotype (Chandipura virus) from India and Nigeria suggest that this group of viruses may have a much wider distribution. Reports of human cases of vesicular stomatitis in the United States and results of immunologic surveys in Central America and Panama indicate that the Indiana and New Jersey strains of VSV also infect man and may be of considerable public health importance. Reported clinical cases of the disease suggest that the virus produces an influenza-like illness in humans. At present, however, little is known about the natural history of the disease. Isolations of VSV-Indiana and Chandipura virus from naturally infected phlebotomine sandflies plus recent studies demonstrating VSV-Indiana transmission by experimentally infected sandflies suggest that these insects may serve as vectors of at least two VSV types. Our recent demonstration of transovarial transmission of VSV-Indiana through two consecutive generations of L. trapidoi is significant, for it provides a mechanism for infection of this species and for maintenance of the virus in nature. It also represents the first conclusive evidence for transovarial transmission of a vertebrate virus vectored by a dipteran insect.

Proposed Course of the Project:

This project is designed to elucidate the ecology of VSV and the epidemiology of the disease. Related entomologic studies are providing considerable new information about the feeding patterns, vector potential, and ecology of New World phlebotomine sandflies.

Our field isolations of a number of Phlebotomus Fever Group viruses from sandflies have also focused our interest on this group of agents which, historically, have been of considerable public health and military importance. Serologic studies of the group are now in progress. By analogy, the VSV-Indiana transovarial transmission model may explain the multiple isolations of Phlebotomus Fever Group viruses from male sandflies and suggests that sandfly-associated viruses do not follow the conventional insect-vertebrate cycle of

mosquito-borne viral agents. If true, this would be of considerable epidemiologic importance. Experiments are now in progress to attempt to prove this hypothesis and to demonstrate transovarial transmission of several Phlebotomus Fever Group viruses by sandflies. During the terminal, contract-supported year available, we also plan to assess other viruses of the VSV complex for transovarial transmission in sandflies.

Publications:

Tesh, R. B., Chaniotis, B. N., and Johnson, K. M.: Vesicular stomatitis virus, Indiana serotype: Multiplication in and transmission by experimentally infected phlebotomine sandflies (Lutzomyia trapidoi). Amer. J. Epidemiol. 93: 491-495, 1971.

Chaniotis, B. N., Neely, J. M., Correa, M. A., Tesh, R. B., and Johnson, K. M.: Natural population dynamics of phlebotomine sandflies in Panama. J. Med. Entomol. 8: 339-352, 1971.

Chaniotis, B. N., Correa, M. A., Tesh, R. B., and Johnson, K. M.: Daily and seasonal man-biting activity of phlebotomine sandflies in Panama. J. Med. Entomol. 8: 415-420, 1971.

Chaniotis, B. N.: Female gynandromorphs of Lutzomyia trinidadensis (Newstead) from Panama. J. Med. Entomol. 8: 459, 1971.

Tesh, R. B., Chaniotis, B. N., Carrera, B., and Johnson, K. M.: Further studies of the natural host preferences of Panamanian phlebotomine sandflies. Amer. J. Epidemiol. 95: 88-93, 1972.

Tesh, R. B., and Johnson, K. M.: Vesicular stomatitis. In Hubbert, W. T., McCulloch, W. F., and Schurrenburger, P. R. (Eds.): Diseases Transmitted From Animals to Man, Sixth Ed., Charles C. Thomas, Springfield, Ill. (in press)

Tesh, R. B., Chaniotis, B. N., and Johnson, K. M.: Vesicular stomatitis virus (Indiana serotype): Transovarial transmission by phlebotomine sandflies. Science. 175: 1477-1479, 1972.

Rutledge, L. C., and Mosser, H. L.: Biology of immature sandflies (Diptera, Psychodidae) at the bases of trees in Panama. Environmental Entomol., 1972 (in press)

Chaniotis, B. N., Tesh, R. B., Correa, M. A., and Johnson, K. M.: Diurnal resting sites of phlebotomine sandflies in a Panamanian tropical forest. J. Med. Entomol., 1972 (in press)

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PHS-NIH
Summary Statement
Laboratory of Microbial Immunity
July 1, 1971 through June 30, 1972

Studies in this Laboratory concern primarily the basic mechanisms involved in cellular and humoral immune responses to microbial and tissue antigens. A special facility is maintained for work with germfree mice. Work on the immune response to type III pneumococcal polysaccharide has been continued; genetically determined high and low responder strains of mice have been identified, and the nature of the genetic controls is being analyzed. Investigations of cell interactions in graft-versus-host (GVH) reactions have demonstrated synergism between blood and thymus cells using a mortality assay, and have indicated that precursors of cells responsible for splenomegaly and those responsible for death are different. It has been possible to obtain from spleen two poorly reactive populations of thymus-derived cells which cooperate in producing GVH reactions. Another type of cellular immune reaction, autoallergic thyroiditis, also appears to be produced by two types of cell. Requirements for vaccination with killed protozoal parasites are under study. Low doses of such vaccines afford the best protection. The importance of immunoglobulin receptors on the precursors of antibody-forming cells is being investigated. An IgM receptor appears early in the ontogeny of this line of cells in germfree mice, and on precursors of cells which engage in primary immune responses in adult conventional mice.

There have been several changes in staff in the past year. Dr. Robert E. Tigelaar, who came to the Laboratory as a Research Associate in 1970, will remain as a staff member. He will spend one year in the laboratory of Dr. N. A. Mitchison at University College, London, on working assignment. Dr. David R. Barthold joined the Laboratory as a Staff Associate this year and is studying immune responses to type III pneumococcal polysaccharide. Dr. Vilho Pasanen is a Visiting Fellow from Finland. He plans to investigate both cell-mediated and humoral immune responses *in vitro*.

ANTIBODY RESPONSE INFLUENCED BY AN X-LINKED GENE

The IgM antibody response to Type III pneumococcal polysaccharide (SSS-III) was assessed in F_1, F_2, and backcrosses progeny derived from high and low responding parental strains of inbred mice. Data were obtained to indicate that a major component involved in the antibody response is X-linked, i.e., carried on the X-chromosome; this component determines responsiveness to SSS-III in an almost quantal or "all-or-none" manner. In addition, other autosomal genes influence the magnitude of the antibody response produced by mice possessing the X-linked gene; these appear to act by regulating the number of cells participating in the antibody response, as well as the rate of antibody synthesis by such cells. Studies are now in progress to obtain more precise information concerning the mechanism by which these X-linked and autosomal genes exert their effects. (Dr. Baker, Dr. Barthold, Mr. Stashak, and Miss Amsbaugh, with Dr. Prescott, Laboratory of Microbiology, NIAID, and Dr. Hansen, Veterinary Resources Branch, Division of Research Services, NIH)

MICE IMMUNIZED WITH KILLED PLASMODIUM BERGHEI

It is known that protective immunity can be induced in rhesus monkeys against Plasmodium knowlesi, but only by mixing the antigen with complete Freund's adjuvant. Effective protection to malaria parasites without adjuvants has not been achieved. Mice were immunized with high and low doses of Plasmodium berghei, killed by heating or with formalin. Serum antibody was detected in mice that received high doses of the antigens, but no protection to challenge with live parasites was induced. In contrast, animals that were immunized with low dosages of antigen had no detectable serum antibody but were protected against a challenge dose of live P. berghei. At least 3 weeks was required for protection to develop. The protection observed with low doses of P. berghei in the absence of antibody suggests that cell-mediated immunity is significant in the induction of resistance to malaria. A state of tolerance to the functional (protective) antigens may be induced when high doses of malarial antigen are used. These studies further indicate that protection to protozoan parasites can be stimulated without the use of adjuvants. (Dr. Finerty and Mr. Evans)

MORE THAN ONE TYPE OF CELL INVOLVED IN THE PRODUCTION OF THYROIDITIS

Experimental allergic thyroiditis, a disease resembling certain forms of human thyroiditis, may be produced in guinea pigs immunized with extracts of guinea pig thyroid emulsified in complete Freund's adjuvant. The disease can be transferred from immunized animals to others by injecting the lymph node cells of the thyroid immunized donors into untreated recipients. Such transfers are equally successful if the cells are irradiated with 4000 R. Further, methotrexate (MTX) can inhibit the development of the disease in actively immunized animals, but does not eliminat the disease if given after it is formed. However, MTX given to the recipients of cells derived from immunized animals prevents the development of thyroiditis The resistance of the effective subgroup of the transferred cells suggests that they need not multiply to induce disease, and indicates that that subgroup may be T cells rather than B cells. The fact that MTX inhibits the development of

disease in recipients of immunized leukocytes indicates that the transferred cells stimulate a population of the recipients' cells in order to produce disease. Together the results suggest that more than one cell type must act in concert to produce this experimental autoimmune disease. (Dr. McMaster)

TWO POPULATIONS OF THYMUS-DERIVED LYMPHOID CELLS OBTAINED FROM SPLEEN

The efficacy of various lymphoid cells in producing GVH reactions was quantitated by measuring the degree of spleen enlargement they produced in newborn F_1 mice. Normal parental spleen cells were injected into lethally irradiated syngeneic recipients: 24 hours later the cells which had migrated either to the lymph nodes or spleens of such mice were tested for their GVH reactivity. Either the spleen-seeking populations or the lymph node-seeking populations by themselves were markedly deficient in producing GVH reactions compared to the normal population from which they were derived. Appropriate mixtures of the two subpopulations, however, restored the reactivity characteristic of a normal peripheral lymphoid tissue. Both subpopulations were shown to carry antigenic determinants characteristic of thymus-derived cells. This study points to a functional heterogeneity within the peripheral pool of thymus-derived cells and indicates that the normal expression of GVH reactivity of a normal peripheral lymphoid tissue involves cooperation between at least two subpopulations of thymus-derived cell which have different migratory properties in irradiated syngeneic recipients. (Dr. Tigelaar and Dr. Asofsky)

DEVELOPMENT OF "B" LYMPHOCYTES SUPPRESSED

It was shown previously that treatment of germfree mice from birth to maturity with purified goat anti-IgM resulted in low serum immunoglobulin levels, low rates of immunoglobulin synthesis, and failure to make an antibody response following immunization with horse ferritin. Further investigation has revealed that spleens from such mice have about 40% fewer cells than spleen from animals treated with goat γ-globulin, goat anti γ_1 or untreated controls. Such spleens contained almost no cells which bound fluorescein-labeled anti-immunoglobulins or horse ferritin labeled with I^{125} either before or after immunization with this antigen. All control groups (goat γ-globulin, goat anti-γ_1, no treatment) had cells which bound ferritin, especially after immunization, and 20-40% of cells bound anti-immunoglobulin each. Most of the ferritin-binding cells in immunized groups (ca. 60%) and almost all of the ferritin-binding cells in unimmunized mice ($>$ 90%) bound anti-IgM. Graft-versu host activity of spleen cells from suppressed mice was at least normal. It was necessary to begin treatment just after birth to obtain suppression. This work indicates that the precursors of cells which synthesize all classes of immunoglobulin and antibody (B lymphocytes) are sensitive during their development to treatment with anti-IgM, presumably because they carry IgM on their membrane (Dr. Asofsky, Dr. Tigelaar, Miss Hylton, Dr. Davie, Laboratory of Pathology, NCI)

Serial No. NIAID - 110
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Administration, research planning and coordination

Previous Serial Number: Same

Principal Investigator: John E. Tobie

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	21/12			21/12
Professional:	12/12			12/12
Other:	9/12			9/12

Project Description:

Administrative and supervisory support is provided to all personnel and to each project of the Laboratory.

Research plans are discussed with the various investigators, over-all evaluations made and the research activities of the Laboratory coordinated. Collaborative research with specialists in other laboratories of NIAID, or institutes of the NIH, or various university investigators is encouraged and assistance given the professional staff in effecting the necessary arrangements. Technical consultation is provided to individuals of the NIH, and to scientists of other departments of the Government or academic organizations. Assistance is provided the professional staff in the preparation of manuscripts and in editing and reviewing a variety of technical reports. The needs of all personnel are evaluated and an effort made to obtain the necessary equipment and supplies for the efficient conduct of research. Budget requirements are forecast on a yearly basis and emergency needs dealt with as problems arise. Supervision is given in regard to laboratory expenditures, travel of personnel, and correspondence. Highly qualified professionals and technicians are sought as staff additions or replacements to insure a high level of research excellence.

Serial No. NIAID - 111
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Response of germfree and conventional experimental animals to inoculation with axenically cultivated Entamoeba histolytica; mechanisms of pathogenesis in experimental amebic enteritis

Previous Serial Number: Same

Principal Investigator: Bruce P. Phillips

Other Investigators: Louis S. Diamond* (Project Subtitle I)
Akio Takeuchi** (Project Subtitle II)

Cooperating Units: * Laboratory of Parasitic Diseases, NIAID
**Department of Pathology, Walter Reed Army Institute of Research, Washington, D. C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	8/12			8/12
Professional:	6/12			6/12
Other:	2/12			2/12

Project Description:

Project Subtitle I: Response of germfree and conventional experimental animals to inoculation with Entamoeba histolytica from axenic cultures in vitro.

Objectives:

To evaluate the effect of axenic cultivation on the infectivity and pathogenicity of Entamoeba histolytica and, if possible, to induce amebic enteritis in experimental animals with these pure cultures of amebae.

Methods Employed:

E. histolytica from axenic culture in Diamond's medium are pooled and inoculated intracecally into germfree and conventional guinea pigs and rats. The animals are sacrificed and examined for lesions and amebae at various post-inoculation intervals.

Major Findings:

Three strains of E. histolytica, which exhibited marked pathogenicity for experimental animals when maintained with their bacterial flora in crude cultures, became avirulent for germfree and conventional guinea pigs and rats following axenic cultivation. This may be due to failure of the amebae to encyst periodically in axenic culture. Efforts to restore virulence to one strain by return to bacterial associates and attempting to induce encystation showed that there was an apparent irreversible loss of virulence and encystment potential in axenic culture, and these were believed to be closely related.

Significance to Biomedical Research and the Program of the Institute:

One of the principal reasons investigators sought the goal of axenic cultivation of E. histolytica was to provide a "pure"source of amebic inocula for animal studies. Data showing loss of ameba virulence in axenic culture point to the need for additional work on axenic cultivation of this ameba with the objective of developing methods that will preserve the pathogenic potential in culture.

Proposed Course of Project:

Plan to test periodically new strains isolated into axenic culture and to test the effects of variations in axenic cultivation techniques, as they are developed, on ability of the amebae to encyst and produce disease when introduced into experimental animals. Also plan to ascertain the effects of intrahepatic inoculation of ameba into hamsters (an accepted method for increasing amebic virulence) on the ability of the amebae to encyst and to produce disease in appropriate hosts.

Project Subtitle II: Mechanisms of pathogenesis in experimental amebic enteritis.

Objectives:

To ascertain the specific mechanisms involved in penetration of the intestinal epithelium by the amebae.

Methods Employed:

Germfree guinea pigs are inoculated intracecally with E. histolytica and its accompanying bacterial flora from acute cases of (human) amebic enteritis. Animals are sacrificed at intervals of from a few hours to two weeks and tissues are prepared for examination by light and electron microscopy.

Major Findings:

The detailed examination of more than one thousand electron micrographs has revealed a distinct and consistent pattern of the invasive process. Close association of ameba with the intestinal epithelium results in degeneration of the brush border and a toxic degeneration of mitochondria in the cellular

constituents. As the ameba moves closer to the degenerating microvilli, the cytoplasm of epithelial cells projects into the lumen, contacting the ameba, thus detaching epithelial cells from their basement membrane and providing open spaces in the epithelial barrier. Amebae begin to enter these spaces and immediately encounter the wandering elements attracted to the damaged areas. The amebae appear to have a lethal effect on these leukocytes causing them to lyse.

Significance to Biomedical Research and the Program of the Institute:

The pathogenic mechanisms in intestinal amebiasis have been the subject of considerable speculation and scientific debate since 1875. Many theories have been proffered on the mechanism of tissue penetration by E. histolytica, none of which could be scientifically supported. This project has provided the first evidence based on fine structural study on the mechanism of tissue penetration by the ameba.

Proposed Course of Project:

Observations thus far have been concerned almost exclusively with the initial mechanism of barrier penetration. We plan to continue the studies to ascertain the mechanism of tissue changes that occur deeper within the intestinal wall. It is planned to add a cyto-chemist collaborator from Dr. Takeuchi staff in an attempt to classify the apparent toxic agent secreted by the amebae

Honors and Awards:

Mr. Phillips was invited to chair the Parasitology Section of the 4th International Symposium on Germfree Research, New Orleans, Louisiana, April 16-20, 1972.

Publications:

Phillips, B. P., Diamond, L. S., Bartgis, I. L., and Stuppler, S. A.: Results of intracecal inoculation of germfree and conventional guinea pigs and germfree rats with axenically cultivated Entamoeba histolytica. J. Protozool. (in press).

Phillips, B. P.: Concurrent irreversible loss of infectivity-pathogenicity and encystment potential in a strain of Entamoeba histolytica after prolonged maintenance in axenic culture in vitro. Exp. Parasitol. (in press).

Serial No. NIAID - 111-C
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Investigations on Blastocystis hominis in germfree and controlled flora experimental hosts

Previous Serial Number: Same

Principal Investigator: Bruce P. Phillips

Other Investigators: Charles H. Zierdt*

Cooperating Units: *Laboratory of Clinical Pathology, CC, NIH, Bethesda, Md.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	3/12			3/12
Other:	1/12			1/12

Project Description:

Objectives:

To ascertain whether Blastocystis hominis will grow in germfree and/or controlled flora animals and, if so, to determine whether it produces symptoms and/or pathology, and to determine what stages in its life cycle may be found in experimental hosts.

Methods Employed:

B. hominis was isolated into culture from patients in the Clinical Center. Our interest was predominantly with strains isolated from patients with dysenteric symptoms but no known pathogenic organisms in the intestine. Antibiotic combinations, following drug sensitivity studies, were utilized to axenize the B. hominis cultures for use in germfree guinea pigs. In some instances, however, the whole untreated culture with the bacterial flora from the patient was introduced into germfree guinea pigs.

Major Findings:

It was possible to inhibit completely the accompanying bacterial flora

without damaging B. hominis by employing combinations of 8 antibiotics and maintaining the cultures under strictly anaerobic conditions. Germfree guinea pigs were inoculated either orally or intracecally with more than 2 x 10^5 B. hominis. The animals remained germfree and did not in any instance develop B. hominis infections. On the other hand, germfree guinea pigs which received B. hominis and the accompanying bacterial flora from the patient became infected by intracecal and oral inoculation. Some very heavy infections were produced in animals which received repeated oral inoculations. Many different stages in the life cycle of the organism were observed in preparations from infected animals, but thus far there has been no evidence of any gross pathology. One animal, however, showed a rather persistent diarrhea and appeared less healthy than some of his litter mates.

Significance to Biomedical Research and the Program of the Institute:

For many years this organism, which seems to possess a variety of protozoan characteristics, has been known as a "harmless yeast" that was, at its worst, a mere nuisance that often inhabited cultures of intestinal protozoa. There is mounting evidence that B. hominis may sometimes cause dysenteric symptoms, and there is no doubt that it has an ameba form in its life cycle that, in fresh preparations, can be mistaken for (other) intestinal amebae. Studies in experimental animals may aid in elucidation of its life cycle and provide experimental data regarding the pathogenic potential of the organism.

Proposed Course of Project:

To continue infecting animals, as in the past, to study stages found in the intestine of experimental animals with light and electron microscopy and to process any possibly involved tissues for evidence of pathological changes.

Honors and Awards: None

Publications: None

Serial No. NIAID - 111-D
1. Microbial Immunity
2. Office of the Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Response of germfree and conventional guinea pigs to intranasal instillation with Mycoplasma sp. from humans

Previous Serial Number: None

Principal Investigator: Bruce P. Phillips

Other Investigators: Helmut Brunner*

Cooperating Units: *Laboratory of Infectious Diseases, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	3/12			3/12
Other:	1/12			1/12

Project Description:

Objectives:

To evaluate the pathogenicity of Mycoplasma sp. from human sources in germfree and conventional animals.

Methods Employed:

Mycoplasma isolated from patients with pleuropneumonia of mycoplasmal etiology is instilled intranasally into germfree and conventional guinea pigs. Animals are sacrificed at weekly intervals following inoculation, and pulmonary tissues are assayed for the organisms and processed for pathological examination.

Major Findings:

Conventional guinea pigs often succumb to an acute pneumonitis, but germfree guinea pigs have thus far been able to withstand the infection. In germfree guinea pigs, however, there is recoverable virus in the nasal and pulmonary tissues, and there are tissue changes evident upon gross observation of the lungs. Histopathologic examination is as yet incomplete but is expected to form a better basis for comparison of the disease process in the germfree

and conventional animals and to indicate what role, if any, bacterial associates in the flora may play in the disease process. Also of interest, the germfree animals become symptomatic of a possible inflammatory process involving either the muscles or bones of the limbs and feet. Appropriate tissues have been taken in an attempt to identify the nature of this unanticipated syndrome.

Significance to Biomedical Research and the Program of the Institute:

The role of Mycoplasma sp. in the causation of infectious disease syndromes is now universally accepted. It is imperative to learn as much as possible concerning the disease-producing-potential of this important group of microorganisms.

Proposed Course of Project:

Project is expected to be of short duration, probably limited to inoculation of several groups of conventional guinea pigs and only enough germfree guinea pigs to get a legitimate appraisal of the effects on this germfree species.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The genetic, cellular, and regulatory factors in autoallergic disease

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: Richard Asofsky, LMI, NIAID
Michael Kyriakos*
Gordon Sharp**

Cooperating Units: *Department of Pathology, Barnes Hospital, St. Louis, Mo.
**Department of Medicine, University of Missouri Medical School, Columbia, Mo.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	26/12			26/12
Professional:	5/12			5/12
Other:	21/12			21/12

Project Description:

Project Subtitle I: Evidence for the existence of inducer and effector cells in the development of autoimmune disease

Objectives:

To elucidate the role of leukocytes in the genesis of experimental allergic thyroiditis.

Methods Employed:

Guinea pigs were immunized with guinea pig thyroid extract and Freund's complete adjuvant, or with an alternative control antigen. Six to 8 days later the cells were harvested and when desirable X-rayed with 4000 R or else lysed with water. The cells were then inoculated IP into histocompatible recipients. Twelve days later their thyroid glands were removed, fixed in formalin, sectioned, and the sections stained with hematoxylin and eosin. Selected

recipients were inoculated IP twice a week with enough methotrexate to prevent the development of allergic thyroiditis by active immunization. Twelve days after the transfer the thyroid glands of the recipients were removed, fixed in formalin, sectioned, and the sections stained with hematoxylin and eosin to determine the frequency and severity of thyroiditis which was composed of a chronic leukocytic infiltrate in the thyroid.

Major Findings:

When 0.8×10^9 leukocytes from donor guinea pigs immunized with guinea pig thyroid are given intraperitoneally to recipients, about one third of the recipients develop minimal allergic thyroiditis. When the cells are irradiated with 4,000 R, the same percent of the recipients develops thyroiditis. By contrast, the constituents of lysed cells does not induce the disease. However, if the recipient is given MTX twice a week, the recipient does not develop thyroiditis.

Significance to Biomedical Research and the Program of the Institute:

The results indicate that several populations of cells may be important in the production of this experimental disease in guinea pigs. Cells so X-rayed that they cannot multiply can induce disease in the recipient, provided that the recipient's cells can multiply. The fact that the stimulating cells function after heavy irradiation suggest they may derive from the thymus. The origin of the cells they stimulate remains to be determined. One may speculate that they are B cells. These results may lead to the identification of the cell types involved in the production of autoimmune diseases, and ultimately to better therapy.

Proposed Course of Project:

To determine the precise cell types which act as stimulator cells in the transfer and which as effector cells in the recipient. For this purpose transfers comparing populations lacking T or B cells will be attempted along with other fractionation methods. Attempts to isolate the effector cells from animal tissues including the inflamed gland will be tried in order to identify their nature.

Project Subtitle II: The effect of hyperimmune rabbit anti-guinea pig thyroglobulin antibody raised by long-term immunization upon the guinea pig thyroid.

Objectives:

To determine the effect of antiserum obtained after prolonged repeated immunization upon the thyroid gland.

Methods Employed:

Repeated injections of guinea pig thyroglobulin were given to rabbits for 2 years. The antiserum collected at the end of that time was absorbed with guinea pig serum and then given every other day to guinea pigs intraperitoneally

The thyroid glands were removed 2 days after the last injection and prepared for histologic examination or cultured with radioactive media to show whether the cells in the glands were producing immunoglobulins, as detected by radioimmunoelectrophoresis.

Major Findings:

The serum used produced extensive infiltrates of the thyroid gland comprised of eosinophiles of two types, not one, and modest infiltrates of lymphocytes and macrophages. The cells infiltrating the glands appear to be actively producing gamma globulin.

Significance to Biomedical Research and the Program of the Institute:

The work shows the existence of two types of eosinophiles. It shows antibody to a target tissue can attract these to the tissue as well as attracting by some means, direct or indirect, lymphocytes and macrophages. The latter appear to be activated in some way so that they are producing globulins.

Proposed Course of Project:

To fractionate the serum to determine which molecules are responsible for attracting each cell type to the target tissue, and to determine if the infiltrate is making specific antibody to the target.

Honors and Awards: None

Publications: None

Serial No. NIAID - 113-C
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Allergic ocular inflammation

Previous Serial Number: 113-C

Principal Investigator: Philip R. B. McMaster

Other Investigators: Samuel B. Aronson*
Thomas Moore*
Vernon G. Wong**

Cooperating Units: *Department of Ophthalmology, University of California School of Medicine, San Francisco, Calif.
**National Eye Institute

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	5/12			5/12
Professional:	3/12			3/12
Other:	2/12			2/12

Project Description:

Objectives:

To evaluate the role of delayed and immediate hypersensitivity in the production of an autoallergic disease, experimental allergic uveitis, and to devise ways of preventing the disease.

Methods Employed:

Guinea pig retinal extract emulsified in Freund's adjuvant is used to immunize guinea pigs and the effects compared with those in other guinea pigs immunized with the retinal extract and bovine gamma globulin. The injections were then repeated 3 weeks later. The eyes of the animals were observed with the aid of a slit lamp. After 8 weeks they were skin tested with retinal extract. Then the eyes were fixed in formalin and sectioned and stained for histologic analysis.

Major Findings:

Large quantities of bovine gamma globulin given along with the retinal antigen prevent the development of immediate and delayed skin sensitivity as well as uveitis. By contrast small quantities of the competing antigen, BGG, did not prevent the development of immediate or delayed skin sensitivity but did inhibit totally the development of allergic uveitis. In addition, giving methotrexate for 3 weeks starting 5 days after immunization can prevent the development of autosensitization and the development of the disease.

Significance to Biomedical Research and the Program of the Institute:

The experiment shows the development of this autoallergic disease may be prevented by immunizing with an antigen other than uvea at the time one immunizes with uvea, thus offering an opportunity to attempt to prevent the development of sympathetic ophthalmia in man by the same means. The fact that certain immunizing combinations may produce intense immediate and delayed sensitivity to uvea without causing uveitis sheds further light upon the pathogenesis of autoallergic disease.

Proposed Course of Project: Terminated

Honors and Awards: None

Publications:

Aronson, S. B., McMaster, P. R. B., Moore, T. E., Jr., and Coon, M. A.: Toxicity of the cyanoacrylates. Arch. Ophthal. 84: 342-349, 1970.

Aronson, S. B., Moore, T. E., Jr., McMaster, P. R. B., and Coon, M. A.: The pathogenesis of suture toxicity. Arch. Ophthal. 84: 641-644, 1970.

McMaster, P. R. B., Moore, T. E., Jr., and Aronson, S. B.: The role of indigenous bacteria in anterior ocular inflammation. Arch. Ophthal. 86: 443-445, 1971.

Aronson, S. B., and McMaster, P. R. B.: Chronic experimental allergic uveitis. Arch. Ophthal. 86: 438-442, 1971.

Aronson, S. B., and McMaster, P. R. B.: Passive transfer of experimental allergic uveitis. Arch. Ophthal. 86: 557-563, 1971.

Wong, V. G., Anderson, R. R., and McMaster, P. R. B.: Endogenous immune uveitis. Arch. Ophthal. 85: 93-102, 1971.

Serial No. NIAID - 113-D
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Effect of lincomycin derivatives upon acute toxoplasmosis in mice

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: John F. Finerty, LMI, NIAID
Kendall Powers*
Milford N. Lunde*

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To improve the therapy of toxoplasmosis in man.

Methods Employed:

Mice are inoculated with virulent toxoplasma organisms. Some are fed daily selected doses of U-21 or U-24 either by gavage or mixed in the feed. The survival is then recorded. Survivors were sacrificed 42 days after infection and their tissues subinoculated into fresh mice to determine if they had been cured or if they harbored a subclinical infection.

Major Findings:

U-21 and U-24 can eradicate an infection induced with 10,000 RH strain trophozoites provided the drug is given within 48 hours of the parasite inoculation. U-24 appears to be more effective than U-21 when compared on the

basis of mg of drug/kg of animal. When given in the feed, the effect of these drugs compares favorably with sulfonamides and pyrimethamine.

Significance to Biomedical Research and the Program of the Institute:

The work indicates that these two drugs may be helpful in the therapy of toxoplasmosis. This disease may account for as much as 44% of chronic granulomatous uveitis in man.

Proposed Course of Project:

To test the effect of these drugs on toxoplasma-induced uveitis.

Honors and Awards: None

Publications: None

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic tolerance

Previous Serial Number: Same

Principal Investigator: Philip R. B. McMaster

Other Investigators: William Paul*
John Finerty, LMI, NIAID

Cooperating Units: *NIAID, Laboratory of Immunology

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	2/12			2/12
Professional:	2/12			2/12
Other:	0/12			0/12

Project Description:

Project Subtitle: The production of immunity and tolerance to pneumococcal polysaccharide and to pneumococcal polysaccharide coupled to bovine gamma globulin.

Objectives:

To determine if pneumococcal polysaccharide can induce tolerance in an animal which does not respond to that material as an antigen, but does respond to it as a haptene.

Methods Employed:

Guinea pigs are injected with a large quantity of pneumococcal polysaccharide. They are then given an injection of an emulsion of pneumococcal polysaccharide coupled to a protein. Other guinea pigs are injected with the polysaccharide or else with an emulsion of the polysaccharide coupled to BGG. Mice are given selected quantities of the polysaccharide or else the complex of protein and polysaccharide and then challenged with an immunizing quantity of polysaccharide. Serum samples are collected at appropriate intervals and then analyzed for antibody by a radioimmuno assay or else by agglutinin tests.

Major Findings:

Large quantities of pneumococcal polysaccharide can produce tolerance in guinea pigs, for such animals do not make any detectable antibody for months afterward, even when challenged with a strong immunizing dose of the polysaccharide coupled to BGG in adjuvant, which would otherwise lead to high titers of antibody. In mice the quantity of polysaccharide coupled to BGG required to cause tolerance is about the same as the amount of pure polysaccharide required to do so.

Significance to Biomedical Research and the Program of the Institute:

The work shows that tolerance may be produced to a complex haptene in an animal that does not respond to that material by making a detectable immune response. The tolerance is long lasting and resists a potent immunizing challenge. Since the immunizing challenge almost certainly requires T and B cell cooperation, the system may permit an analysis of the role of these cells in the induction of long lasting tolerance, which could be important in the development of better treatment of many diseases.

Proposed Course of Project:

To determine the role of T and B cells in this form of tolerance.

Honors and Awards: None

Publications: None

Serial No. NIAID - 116
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunoglobulin formation in germfree mice

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Martha B. Hylton, LMI, NIAID
Joseph M. Davie*
Michael Potter**
Elizabeth B. Mushinski**
Alexander R. Lawton, III***
Max D. Cooper***

Cooperating Units: *Laboratory of Pathology, NCI
**Laboratory of Biology, NCI
***University of Alabama School of Medicine, Birmingham, Al

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	38/12			38/12
Professional:	8/12			8/12
Other:	30/12			30/12

Project Description:

Objectives:

To study the response of germfree animals to a variety of immunologic stimuli with respect to levels in serum and relative rates of synthesis of various classes of immunoglobulins.

Methods Employed:

Immunoglobulin levels are measured by radial immunodiffusion using agarose plates impregnated with monospecific antisera. Biosynthesis is studied by incubating tissues in vitro in a medium containing C-14 labeled amino acids. Media are then tested for newly synthesized proteins by means of radioimmunoelectrophoresis. Antibodies are measured by passive hemagglutination, by use of specific immunoadsorbents, and by a complement-dependent bactericidal assay.

Plasma cell tumors are induced by inoculation of light mineral oil or pristane intraperitoneally. Immunoglobulin synthesis is suppressed by treating mice from birth with goat anti-mouse IgM. Mice are also treated with anti-γ_1 or normal goat γ-globulin.

Major Findings:

Sera from unimmunized germfree mice contain only 7Sγ_1 and IgM. Immunization with ferritin or other proteins results in a transient increase in IgM and a sustained increase in 7Sγ_1. A second injection of the same (but not another) antigen results in a sustained increase in IgM levels, further increases in 7Sγ_1, and the first appearance of 7Sγ_2. Specific antibody accounts for less than 10% of the IgM, less than 20% of the 7Sγ_1, and less than 40% of the 7Sγ_2. Increasing the interval between the first and second injection of antigen results in increased antibody titers, with no increase in the fraction of each immunoglobulin that can be accounted for as specific antibody.

Intraperitoneal inoculation of an endotoxin results in a sustained increase in IgM with doses of antigen between 5 x 10^{-4} µg and 50 µg. IgM and bactericidal levels are well correlated, but less than 10% of the IgM is specific antibody. Inoculation of a "native protoplasmic polysaccharide" from E. coli, a material with molecular weight about 110,000, results in increases in IgM levels comparable to those seen with endotoxins.

Germfree Balb/c mice inoculated with light mineral oil or pristane develop very few plasma cell tumors in contrast to the high incidence obtained in conventional animals. Germfree Balb/c mice monocontaminated with bacteria or fed dead bacteria, then inoculated with mineral oil or pristane develop plasma cell tumors in much higher incidence than do uncontaminated mice, but in lower incidence and much later than conventional mice. All of the tumors produced by monoinfected germfree mice that have been examined thus far produced IgA myeloma proteins. Mineral oil or pristane granulomas synthesized small amounts of all classes of immunoglobulins within one week of inoculation. There is an incidence of reticulum cell sarcoma of about 15% in all aged (> 18 months) GF Balb/c mice.

Treatment of germfree mice from birth with goat anti-IgM results in reduction or elimination of all immunoglobulins, especially IgM, in the circulation. Mice treated with goat γ-globulin have elevated immunoglobulin levels and circulating antibodies to goat serum proteins; mice treated with anti-IgM lack such antibodies and have residual heterologous anti-IgM in the circulation. These animals do not make antibody responses to new antigens. Their lymphoid tissues lack germinal centers, Peyer's patches, and cells capable of binding antibodies to immunoglobulins. Cells from these animals produce at least normal GVH reactions. Treatment with anti-γ_1, small amounts of anti IgM, or failure to begin treatment early produce no suppressive effect.

Significance to Biomedical Research and the Program of the Institute:

Most immunoglobulin synthesis in the immunized germfree animal is a result of experimental manipulation. Stimulated synthesis of immunoglobulins, especially of IgM, does not represent antibody synthesis. The increase with time

in the fraction of the increased immunoglobulins that are specific antibody is reminiscent of the increases of affinity of antihapten antibodies with time, and probably represents a continued selection of "more specific" cells.

Antigenic stimulation via the gut seems to be important for the development of plasma cell tumors. Increasing numbers of plasma cell tumors have been shown to have defined antibody activity. Studies of plasma cell neoplasia with limited antigenic stimuli should help in the understanding of the pathogenesis of this type of tumor.

Mice treated with anti-IgM show profound hypogammaglobulinemia. The condition produced by this type of treatment resembles certain kinds of human agammaglobulinemia, and may serve as a model for the study of this disease.

Proposed Course of Project:

Plasma cell tumor induction will be attempted in germfree mice fed endotoxins, protein antigens, or killed bacteria. Animals treated with anti-IgM will be immunized with a variety of antigens in adjuvants. Germfree mice will be treated with anti-γ_2 and anti-IgA.

Honors and Awards: DHEW Superior Service Award
Arthur S. Flemming Award

Publications:

Lawton, A. R., Asofsky, R., Hylton, M. B., and Cooper, M. D.: Suppression of immunoglobulin class synthesis in mice. I. Effects of treatment with antibody to μ chain. J. Exp. Med. 135: 277-297, 1972.

Pierce, C. W., Solliday, S., and Asofsky, R.: Immune responses in vitro. IV. Suppression of primary γM, γG, and γA plaque-forming cell responses in mouse spleen cultures by class-specific antibody to mouse immunoglobulins. J. Exp. Med. 135: 675-697, 1972.

Pierce, C. W., Solliday, S., and Asofsky, R.: Immune responses in vitro. V. Suppression of γM, γG, and γA plaque-forming cell responses in cultures of primed mouse spleen cells by class-specific antibody to mouse immunoglobulins. J. Exp. Med. 135: 698-710.

Serial No. NIAID - 116-J
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The suppressive effects of heterologous antithymocyte sera on the homograft reactions in mice

Previous Serial Number: Same

Principal Investigator: Richard Asofsky

Other Investigators: Robert E. Tigelaar, LMI, NIAID
Harvey I. Cantor*
Marek Cwynarski**

Cooperating Units: *National Institute for Medical Research, London, England
**Immunology Branch, NCI

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	28/12			28/12
Professional:	4/12			4/12
Other:	24/12			24/12

Project Description:

Objectives:

To investigate the mechanism of action of antithymocyte sera in suppressing graft-versus-host reactions and allograft rejection.

Methods Employed:

Antithymocyte sera (ATS) were prepared by immunization of rabbits with two intravenous injections of Balb/c thymus cells. Graft-versus-host assays are performed by inoculating spleen cells from mice into newborn allogeneic recipients, and measuring the resulting splenomegaly 9 days later. The activity of cells from animals receiving different doses of ATS was measured with a GVH assay 2 days after treatment. In addition, the capacity of blood lymphocytes (PBL) to potentiate the activity of normal thymus cells was measured (see NIAID-117). In cases in which the dose of ATS was sufficient to suppress the activity of spleen cells, restoration of the activity of these cells by the addition of a small number of PBL was attempted. The effect of ATS on GVH reactivity is studied using a death assay (see NIAID - 117) as well. Finally, the effects

of ATS on interacting cells from germfree (GF) mice are examined. The response of mice treated with ATS to sheep erythrocytes is assayed by counting antibody-forming cells using the Jerne plaque assay.

Major Findings:

Pretreatment of donors with 0.05 ml of ATS produces less than a 20% reduction in the GVH activity of PBL, but produces an 80-90% reduction in the capacity of these cells to interact synergistically with thymus cells. Treatment of donors with 0.25 ml of ATS reduces the GVH activity of spleen cells by more than 80%, but these cells can be restored to almost normal reactivity by addition *in vitro* of 10^5 normal PBL. Treatment with 0.5 ml ATS produces a slightly greater decrease in activity of spleen cells, but these cells can be restored only slightly by the addition of normal PBL. Moderate doses of ATS reduce the activity of cells from treated GF mice measured directly; spleen cells from such mice are restored poorly by addition of normal PBL.

Pretreatment of ATS-treated donors by adult thymectomy (Tx) significantly impairs the recovery of the direct GVH reactivity of spleen, PBL, or LN cells seen in mice treated with 0.25 ml ATS alone. However, spleen cells from Tx-ATS-treated donors, which had less than 10% normal reactivity at 3 days had about 25% normal reactivity by 30 days. There appeared to be little further recovery after 30-60 days: 20 days after treatment spleen cells still exhibit about 25% normal reactivity. At all times studied the reactivity of treated spleen cells could be increased by the addition of small numbers of normal PBL. The ability of PBL from either ATS-treated or Tx-ATS-treated donors to augment the activity of normal thymus cells was markedly suppressed at 3 days. However, this activity was enhanced (i.e., up to 108% of normal) at 11-18 days in both groups, in spite of the fact that the direct GVH reactivity of such PBL was markedly suppressed.

The effect of ATS on the antibody response to sheep erythrocytes is identical in GF and conventionally reared mice.

Significance to Biomedical Research and the Program of the Institute:

ATS is a potent and useful immunosuppressant which has found important clinical use. This study shows that 2 cell components required for GVH activity are differently susceptible to heterologous ATS; rather large doses of ATS are required to suppress both cell types. It is probable that more effective suppression can be obtained by combining ATS with treatment that affects primarily the cells least susceptible to ATS.

The slight but detectable recovery with time of direct GVH reactivity of cells from donors pretreated with thymectomy and ATS may indicate the existence of a population of peripheral thymus-derived cells not eliminated by a moderate dose of ATS which are capable of expansion and/or differentiation in the absence of a thymus. Alternatively, the results could be explained by postulating that part of the immunosuppressive effects of ATS are due to blocking of antigen receptors on such cells and not to actual cell elimination: with time as ATS is removed from these sites, the cells can again display reactivity. The

appearance of enhanced amplifying activity in PBL at a time when direct activity is suppressed in Tx-ATS-treated donors may reflect peripheral differentiation to an amplifier cell from an ATS-insensitive precursor. This discrepancy between direct and amplifying activity represents the most convincing data to date that the interaction between subpopulations of thymus-derived cells in producing GVH reactions (see NIAID - 118) is an obligatory one.

Proposed Course of Project:

The remarkably high susceptibility of germfree mice to ATS will be examined further for sensitivity of the individual cell components involved in synergy. The rates of recovery, from a large dose of ATS, of each of the interacting cell components in spleen and the circulation will be determined. The effects of ATS on the thymus will be tested for their capacity to suppress the cells that are relatively resistant to ATS. The effects of repeated small and large doses of ATS on the GVH potential of cells from thymectomized animals will be evaluated.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The immunologic response of conventional and germfree mice to blood-induced infections of Plasmodium berghei

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	30/12			30/12
Professional:	6/12			6/12
Other:	24/12			24/12

Project Description:

Objectives:

To immunize mice against Plasmodium berghei. To determine the dose of malarial antigen that will induce protective immunity in mice. To determine the time interval between antigen injection and parasite challenge that will still yield protection.

Methods Employed:

P. berghei parasites were removed from their erythrocytes and inactivated with formalin treatment or heat. The treated parasites were injected intraperitoneally, without adjuvants, either as a single dose or given multiple doses. Serum antibody was measured by the indirect fluorescent antibody technique. Non-immunized and immunized mice were challenged at various intervals with live P. berghei.

Major Findings:

Mice given large doses of P. berghei antigen had detectable serum antibody whereas animals that received low doses did not. Animals immunized with

large doses of antigen did not survive a challenge infection, while those immunized with low doses did survive. The results indicated that formalin-treated parasites given in low doses were more efficacious in producing protection than higher doses. Heat-inactivated parasites were effective in stimulating protective immunity when given either in high or low doses, provided the challenge with live P. berghei was delayed for at least 5 weeks after immunization. Challenging the mice less than 5 weeks revealed protection in mice that had received the lower doses of heat-inactivated P. berghei, but not high doses.

Significance to Biomedical Research and the Program of the Institute:

The ability to induce protective immunity against malaria without the use of adjuvants indicates that the antigen(s) involved are not destroyed during inactivation. The lack of protection seen in mice immunized with high doses of antigen suggests that these dosages may: (1) induce the synthesis of serum antibody of low avidity; (2) induce tolerance to the functional antigen(s). In the first case, the antibody would be ineffective in protecting the host. The second possibility means there will be no protective antibody synthesized in the host. Finally, (3) it is possible that low doses of antigen induce cellular immunity more effectively, and that this is more important than humoral immunity in providing protection.

Proposed Course of Project:

Future studies will involve the identification and isolation of the functional antigen(s) involved in malarial immunization.

Honors and Awards:

Publications: None

Finerty, J. F., Tobie, J. E., and Evans, C. B.: Antibody and immunoglobulin synthesis in germfree and conventional mice infected with Plasmodium berghei. Am. J. Trop. Med. & Hyg. (in press).

Hyde, C. L., Finerty, J. F., and Evans, C. B.: Antibody and immunoglobulin synthesis in germfree and conventional mice infected with Eperythrozoon coccoides. Am. J. Trop. Med. & Hyg. (in press).

Serial No. NIAID - 117-B
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic studies in simian malaria

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID
Louis Miller*
Kendall Powers*

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID-131

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To study the immune responses of rhesus monkeys (Macaca mulatta) to the simian malaria parasites, Plasmodium knowlesi and P. cynomolgi, as well as owl monkeys (Aotus trivirgatus) to Plasmodium falciparum. To determine the kinetic of the antibody response in blood and sporozoite-induced infections.

Methods Employed:

Rhesus monkeys were infected with P. knowlesi by inoculation of parasitize blood. Parasites were collected at the peak of parasitemia. Other infected monkeys were given doses of chloroquine sufficient to cure the infection; a third group of monkeys were given subcurative doses of chloroquine. These treated monkeys were bled at various times and their sera analyzed for the presence of malarial antibody. Antibody was assayed by the indirect fluorescent antibody technique (IFA) and the schizant-agglutination test (SAT). (See NIAID-131.)

Major Findings:

Monkeys infected with P. knowlesi had low antibody titers as detected by IFA. These titers did not exceed 1:256. In contrast, titers exceeded $1:5x10^5$ when sera were assayed by the SAT. The titers to P. knowlesi were quite low 7 days after inoculation when assayed with schizant agglutination, but increased rapidly by 14 days after inoculation ($1:1x10^5$). Treated monkeys, either those cured or those in which the parasites still remained, revealed little differences in SAT titers 30 days after inoculation.

Significance to Biomedical Research and the Program of the Institute:

The use of the schizant-agglutination technique is an important addition to the study of malarial immunity. The results indicate that the test is reproducible, sensitive, resulting in higher titers than IFA, and is easy to perform. This suggests that the SAT could lend itself to field studies.

Proposed Course of Project:

Future studies will determine the type of antibody detected by the SAT. These studies will atempt to correlate SAT titers in immunized animals to the degree of protection.

Honors and Awards: None

Publications: None

Serial No. NIAID - 117-C
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Separation of different erythrocytic stages of Plasmodium berghei and P. knowlesi

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID
Kendall Powers*
Louis Miller*

Cooperating Units: *Laboratory of Parasitic Diseases, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	4/12			4/12
Professional:	2/12			2/12
Other:	2/12			2/12

Project Description:

Objectives:

To isolate the various erythrocytic stages of Plasmodium berghei, P. knowlesi, and P. falciparum. To concentrate and isolate these stages and use them in the studies of the immune response to malaria.

Methods Employed:

Whole blood parasitized with P. berghei was obtained from BALB/c mice. P. knowlesi was obtained from infected rhesus monkeys at peak parasitemia. Parasites were separated on discontinuous density gradients and then freed from their erythrocytes by ammonium chloride lysis. The parasites were stained with Giemsa solution for microscopic examination. Parasites were inactivated with formalin and utilized to develop a schizant-agglutination test. (See NIAID - 131.)

Major Findings:

Microscopic examination of parasites treated with ammonium chloride revealed intact parasites that retained their characteristic staining morphology. Parasites were observed to be adhering to lymphocytes (rosettes). These rosettes were seen with P. falciparum, P. knowlesi, and P. berghei. Parasitized erythrocytes did not form rosettes with lymphocytes. Only those parasites freed of their erythrocyte membranes as observed by electron microscopy were attached to lymphocytes. Rosettes were not formed with normal lymphocytes. Immune sera did not confer rosette-forming capacity on normal lymphocytes. The number of rosettes increased during acute infection and diminished during recovery.

Fractions of parasitized erythrocytes containing only schizants could be obtained by separation on albumin gradients. These parasites were freed from erythrocytes with ammonium chloride and inactivated with formalin. They gave excellent results when utilized in the schizant-agglutination test (SAT). This was particularly evident with P. knowlesi. Storage of the inactivated parasites at 10C for 6-7 weeks did not alter their antigenic properties in the SAT.

Significance to Biomedical Research and the Program of the Institute:

The rosette formation by freed malarial parasites indicates that the parasites retain their antigenic properties after being freed from their erythrocytes. The results further suggest that rosette-forming cells may detect malarial antigen or antibody not detected by other serologic procedures. This procedure should be useful for detecting "memory cells" in individuals previously exposed to malarial parasites and perhaps correlate these findings with the individual's state of resistance. Further, rosette formation will be useful in detecting the functional antigen(s) that are responsible for eliciting protective host immunity.

Inactivation of the parasites with formalin now adds a new dimension to the immunologic study of host response to malaria. The SAT can be used in conjunction with indirect fluorescent antibody techniques to measure not only antibody synthesis but also the immunoglobulin class of the antibody(s) synthesized. In addition, formalinized parasites used in the SAT should lend itself quite easily to field studies without the use of expensive optical equipment.

Proposed Course of Project:

Future studies with the rosette technique will focus on the types of lymphocytes involved and the immunoglobulin on their surfaces. The various stages of the malarial parasite will be studied to determine if one stage forms rosettes with a greater frequency than other stages.

Parasites treated with formalin will be used to study the kinetics of the immune response to malaria and compare the results to the indirect fluorescent antibody procedure. Attention will focus primarily on antibody produced in actively immunized and protected animals, then compared to those animals that recover naturally from malaria.

Honors and Awards: None

Publications:

Martin, W. J., Finerty, J., and Rosenthal, A.: Isolation of Plasmodium berghei (malaria) parasites by ammonium chloride lysis of infected cells. Nature 233: 260-261, 1971.

Serial No. NIAID - 117-E
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The immunologic response of animals immunized with trypanosomes

Previous Serial Number: Same

Principal Investigator: John F. Finerty

Other Investigators: Charles B. Evans, LMI, NIAID

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	6/12			6/12
Professional:	2/12			2/12
Other:	4/12			4/12

Project Description:

Objectives:

To determine the immunoglobulins produced and classes of antibody synthesized in mice, rats, and rabbits immunized with various preparations of Trypanosoma rhodesiense and T. brucei. To determine the efficacy of the immunization procedures by challenge with live T. brucei and T. rhodesiense.

Methods Employed:

Rabbits were injected with live T. rhodesiense. Infected rabbits were subsequently injected intramuscularly with various antigenic preparations of T. rhodesiense (formalinized, heat-inactivated, or fragmented under pressure). Serum antibody to T. rhodesiense was measured by trypanosomal agglutination and indirect fluorescent antibody technique (IFA). The duration of protective immunity was assayed by challenge with live trypanosomes.

Major Findings:

Rabbits infected with T. rhodesiense developed lesions around the eyes, nose, mouth, and genitals and died within 6-8 weeks after inoculation. Parasites were not readily demonstrable in the peripheral circulation but were abundant in the lesions. Injection of antigenic preparations into infected

rabbits led to recovery. Antibody titers assayed by IFA rose 8-fold within 7 days after injection of the antigens, whereas titers did not increase in non-treated animals. Animals given the antigens remained free of infection for at least 6 months and were immune to challenge with live T. rhodesiense at the end of this time.

Significance to Biomedical Research and the Program of the Institute:

Active immunization of animals infected with trypanosomes has not previously been demonstrated. The data indicate that various antigenic extracts of T. rhodesiense are effective in eliciting a protective immune response in animals already infected. Further studies should define the type of antibody formed or the development of cellular immunity during infection or after immunization.

Proposed Course of Project:

Future studies will focus on the antigen(s) involved in the induction of a protective immune response. The roles of humoral and cellular immunity will be studied in relation to infection and active immunization.

Honors and Awards: None

Publications: None

Serial No. NIAID - 118
1. Microbial Immunity
2. Experimental Pathology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Synergy in the graft-versus-host response

Previous Serial Number: Same

Principal Investigator: Robert E. Tigelaar

Other Investigators: Richard Asofsky, LMI, NIAID
Richard Gershon*
Harvey I. Cantor**

Cooperating Units: *Department of Pathology, Yale University, New Haven, Conn.
**National Institute for Medical Research, London, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	15/12			15/12
Professional:	12/12			12/12
Other:	3/12			3/12

Project Description:

Objectives:

To characterize subpopulations of lymphoid cells which interact in the graft-versus-host (GVH) reaction and to elucidate the nature of this interaction.

Methods Employed:

GVH reactions were produced by inoculation of mouse lymphoid cells from tissues such as spleen, lymph nodes (LN), thymus, and peripheral blood (PBL) or combinations of such cells into newborn allogeneic or F_1 recipients. The degree of GVH activity was assayed by two methods. The Simonsen spleen weight assay, in which the reactivity of the injected lymphoid cells is measured by the degree of spleen enlargement in recipient mice was routinely used. A death assay, in which the cumulative mortality of sublethally irradiated recipients of lymphoid cells was expressed as a function both of time and of inoculum size was also employed. Either normal or thymectomized donors of lymphoid cells, or cell suspensions in vitro, were treated with a variety of agents with affinity for cell membrane constituents (such as anti-thymocyte serum (ATS), anti-theta,

anti-TL, cortisone acetate) and the cells then examined for alteration in their behavior in GVH reactions.

Major Findings:

Studies utilizing the spleen weight assay have indicated that optimal expression of GVH reactions may involve interaction among two or more cell types. These populations have the following characteristics: (1) One population is present in high concentrations in tissues which are rich in recirculating lymphocytes, such as PBL or LN. The second is a population of relatively more sessil cells found in higher concentrations in thymus and spleen. (2) Both population must have the capacity to recognize foreign antigens in the recipient. (3) Both of the cell types involved are "thymic-dependent." (4) The cell type present in excess in thymocyte suspensions appears to act as a precursor of cells which inflict immunologic injury while the cell present in excess in PBL or LN amplifies the activity of the precursor cells.

These findings have been confirmed using the death assay. PBL, the most active cells, were about 30 times as active as thymocytes, the least active cells studied; LN lymphocytes and spleen cells were about 23 and 8 times as reactive as thymocytes, respectively. Mixtures of numbers of PBL and thymus cells which by themselves produced little or no death consistently gave very high levels of mortality by 35 days. The average survival time of recipients of thymocytes who eventually died was nearly a week longer than than of recipients of comparably lethal numbers of PBL, LN, or spleen cells. The kinetics of cumulative mortality observed for mixtures of PBL and thymus cells were indistinguishable from those seen with thymocytes alone, indicating activation of the latter cell type. Both populations had to be able to recognize foreign antigens in the recipient in order for synergy to be seen. Finally, quantitative comparison of the relative abilities of different cell populations to cause splenomegaly on the one hand and lethal runting on the other has raised the possibility that the expression of different effector functions of cell-mediated immune reactions may be initiated by distinct cells.

A series of experiments were designed to see whether manipulation of normal spleen cells could lead to a more explicit demonstration of the existence of subpopulations of cells important in GVH reactions. Normal parental spleen cells were injected into lethally irradiated syngeneic recipients; 24 hours later the spleens and lymph nodes were removed and these populations tested for their GVH reactivity in a spleen weight assay either directly or combined with each other. Spleen-seeking spleen cells (SSSC) were less reactive than normal spleen cells as indicated both by the greater number of such cells required to produce significant minimum splenomegaly as well as by a significantly flatter dose-response curve, i.e., many more SSSC than normal spleen cells were required to produce a given increment in observed splenomegaly. Lymph node-seeking spleen cells (LSSC) also showed a significantly flatter dose-response curve than either normal lymph node or normal spleen cells. Appropriat combinations of SSSC and LSSC produced splenomegaly greater than expected from summing the reactivities of these populations measured separately. Furthermore, these mixtures restored the steep dose-response curve characteristic of normal spleen cells, the population from which they were derived. As in the previous models, substituting syngeneic F_1 cells for either of the passaged parental cell

types did not result in synergy. Pre-treatment of either of the populations in the mixture of parental cells with anti-theta antiserum and complement resulted in levels of splenomegaly similar to those expected from the untreated population alone. Furthermore, SSSC interacted synergistically with appropriate numbers of normal LN cells as did LSSC when combined with normal thymus cells. These data indicate that within the spleen there exist at least two subpopulations of thymus-derived cells which have different migratory properties in irradiated syngeneic recipients, and that both of these cells are necessary for normal and full expression of the GVH reactivity of a normal peripheral lymphoid tissue.

The effect of delaying the injection of either one of the populations of normal lymphoid cells which when injected simultaneously resulted in synergy was investigated. Injection of LN cells 24 hours after injection of thymocytes resulted in spleen indices similar to those seen when these populations were injected together. However, injecting thymocytes 24 hours after LN cells resulted in markedly reduced spleen indices--little synergy was seen.

Thymus cell donors were pretreated with 2.5 mg cortisone acetate 48-72 hours prior to sacrifice. Although the specific activity of such cells was markedly increased compared to normal thymocytes, i.e., reactivity similar to that observed with normal spleen cells, nevertheless only approximately 50% of the total reactivity present in a normal thymus could be accounted for by the reactivity of the small number of cells recoverable from the thymus of a cortisone-treated donor. Furthermore, this population of cells were incapable of interacting synergistically with either normal LN cells or with normal thymocytes, indicating that the population of cells in normal thymus which can interact with LN cells or PBL are sensitive to the action of cortisone acetate, and that the cortisone-resistant cells do not contain an excess of cells capable of amplifying the activity of normal thymus cells.

Rather similar results have been obtained following incubation of thymocytes from an appropriate strain of mice with complement and an antiserum directed against an antigen (TL) present on the majority of such thymocytes. The residual population had enhanced specific activity when tested directly, but had lost its capacity to interact synergistically with normal PBL; combination of these cells with normal thymus cells resulted in recipient splenomegaly only slightly greater than expected, i.e., little if any synergy was observed.

Significance to Biomedical Research and the Program of the Institute:

The present studies have shown that different populations of thymus-derived lymphoid cells can interact synergistically in producing GVH reactions quantitated either by mortality or by splenomegaly. This points to a previously unrecognized functional heterogeneity within the peripheral pool of cells involved in cellular immune reactions against foreign tissues. Further delineation of the nature of this interaction and the characterization of the cells involved is obviously necessary for a clearer understanding of this type of immune response. Appropriate adjustments of the cell populations involved in cell-mediated immune responses against tumor antigens might enhance the process of tumor rejection of either syngeneic or autochthonous tumors. Similarly, the

elimination of small subpopulations of lymphocytes necessary for full expression of normal GVH reactivity may have significance in dealing with the problem of suppression of homograft rejection, and of the graft-versus-host reactions which complicate bone marrow transplantation.

Proposed Course of Project:

To further investigate the relationship of various cell membrane constituents such as theta and TL antigens and the susceptibility to cortisone treatment of thymocytes or thymus-derived cells to the physiologic properties of those cells. To study the effect of adult thymectomy or thymectomy plus treatment with ATS on the capacity of cells to interact in the GVH reaction (see 116-J). To test the capacity of various cell populations alone and in combination to reject or prevent growth of SV-40-induced tumors in mice. To determine if the cell responsible for amplification of GVH reactions has physiologic properties similar to the thymus-derived "helper" cell involved in many humoral antibody responses.

Honors and Awards: None

Publications:

Asofsky, R., Cantor, H., and Tigelaar, R. E.: Cell interactions in graft-versus-host response. In Proceedings of the First International Congress of Immunology. New York, Academic Press, 1971, pp. 369-381.

Cantor, H., and Asofsky, R.: Synergy among lymphoid cells mediating the graft-versus-host response. III. Evidence for interaction between two classes of thymus-derived cells. J. Exp. Med. 135: 764, 1972.

Tigelaar, R. E., and Asofsky, R.: Synergy among lymphoid cells mediating the graft-versus-host response. IV. Synergy in the GVH reaction quantitated by a mortality assay in sublethally irradiated recipients. J. Exp. Med.(in press).

Serial No. NIAID - 119-A
1. Microbial Immunity
2. Microbiology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Quantitative and qualitative studies on the antibody response to pneumococcal polysaccharides at the cellular level

Previous Serial Number: Same

Principal Investigator: Phillip J. Baker

Other Investigators: David R. Barthold, Philip W. Stashak, and Diana F. Amsbaugh, LMI, NIAID
Benjamin Prescott*
Carl T. Hansen**

Cooperating Units: *Laboratory of Microbiology, NIAID
**Veterinary Resources Branch, Division of Research Services

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total:	62/12			62/12
Professional:	24/12			24/12
Other:	38/12			38/12

Project Description:

Project Subtitle I: Genetic control of the antibody response to Type III pneumococcal polysaccharide (SSS-III)

Objectives:

To characterize those genetic factors that regulate the magnitude of the antibody response to SSS-III and to obtain information concerning their mode of action.

Methods Employed:

Several strains of inbred mice were evaluated with respect to their ability to respond to SSS-III. F_1, F_2, and backcross generations derived from crosses between low and high responding strains were prepared and used in studies on the genetic control of responsiveness to SSS-III. An adaptation of the technique of localized hemolysis-in-gel was used to detect antibody-producing or plaque-forming cells (PFC) specific for SSS-III, and serum antibody was

detected by passive immune hemolysis; sheep erythrocytes, sensitized with SSS-III by a chromium chloride coupling procedure, were used in both immunological tests.

Major Findings:

Several strains of inbred mice were found to differ widely in their ability to respond to SSS-III. No relationship between H-2 histocompatibility type and the magnitude of the PFC or serum antibody response produced was evident. Genetic studies conducted with high responding and extremely low responding parental strains revealed that an X-linked gene plays a decisive role in determining responsiveness to SSS-III. Additional autosomal genes influence the magnitude of the antibody response produced by mice possessing the X-linked gene. The latter appear to regulate both the number of cells participating in the antibody response and the rate of antibody synthesis by such cells.

Significance to Biomedical Research and the Program of the Institute:

Several immunological deficiency diseases of man have been reported to be X-linked. In some instances, the ability to respond to polysaccharide antigens, such as SSS-III, is greatly impaired. The above studies may provide a suitable experimental model for investigating such conditions.

Proposed Course of Project:

Additional genetic studies are now being conducted to obtain information concerning the mode of action of the X-linked and autosomal genes described above. These studies are part of a continuing investigation of factors that influence or modify the ability of bone marrow-derived precursors of antibody-forming cells to respond to well defined antigens. SSS-III has an exceedingly simple structure, and induces a brief IgM antibody response, apparently by direct stimulation of "B" lymphocytes. It is therefore an ideal antigen for these studies.

Honors and Awards: None

Publications:

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. I. Dose-response relationships and the effect of prior immunization of the magnitude of the antibody response. Immunol. 20: 469-480, 1971.

Baker, P. J., Stashak, P. W., Amsbaugh, D. F., and Prescott, B.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. II. Studies on the relative rate of antibody synthesis and release by antibody-producing cells. Immunol. 20: 481-492, 1971.

Baker, P. J., Prescott, B., Barth, R. F., Stashak, P. W., and Amsbaugh, D. F.: Immunological paralysis to Type III pneumococcal polysaccharide as assessed by an immuno-plaque procedure. Ann. N. Y. Acad. Sci. 181: 34-46, 1971.

Baker, P. J., Prescott, B., Stashak, P. W., and Amsbaugh, D. F.: Characterization of the antibody response to Type III pneumococcal polysaccharide at the cellular level. III. Studies on the average avidity of the antibody produced by specific plaque-forming cells. J. Immunol. 107: 719-724, 1971.

Barth, R. F., Baker, P. J., Stashak, P. W., and Amsbaugh, D. F.: Enhanced antibody production to Type III pneumococcal polysaccharide in mice treated with antilymphocyte serum: evidence for cellular regulatory mechanism of IgM antibody response. Transpl. Proc. 3: 800-802, 1971.

Project Subtitle II: Regulation of the antibody response to SSS-III by thymic suppressor cells.

Objectives:

To investigate the mechanism by which antithymocyte serum (ATS) enhances the antibody response to SSS-III.

Methods Employed:

The immunological procedures used to detect serum antibody and antibody-producing cells specific for SSS-III are given in the preceding section of this report. Burro or rabbit antimouse thymocyte serum was prepared by a conventional method and administered at the time of immunization with SSS-III; the magnitude of the serum antibody response was assessed 5 days later.

Major Findings:

Treatment with ATS prepared in two animal species (burros and rabbits) resulted in a significant increase in the PFC and serum antibody response to SSS-III in several strains of inbred mice. Such enhancement was demonstrable within a 10-fold dose range using either burro or rabbit ATS; the dose of ATS giving maximal enhancement, as well as the degree of enhancement achieved, varied with the type of ATS and the strain of mouse employed. Treatment with ATS was most effective in enhancing the antibody response when given at the time of immunization.

Significance to Biomedical Research and the Program of the Institute:

Differences noted among strains of inbred mice with respect to both the ability to respond to SSS-III and the degree to which their antibody response is enhanced by treatment with ATS may be related to differences in the number, proportion, and specific activity of amplifier and suppressor cells present. This work raises the possibility that genetic control of immune responses is expressed through both amplifier and suppressor activities of thymus-derived cells.

Proposed Course of Project:

Studies are in progress to determine whether differences between strains of inbred mice in their ability to respond to SSS-III, as well as the degree

of enhancement produced following treatment with ATS, are related to differences in the number and specific activity of amplifier and suppressor cells present. This will be done in conjunction with genetic studies on mice producing high and low antibody responses to SSS-III.

Honors and Awards: None

Publications: None

LABORATORY OF PARASITIC DISEASES

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Laboratory of Parasitic Diseases

National Institute of Allergy and Infectious Diseases

Summary statement of research activities July 1, 1971 through June 30, 1972

INTRODUCTION

Administratively imposed "grade reduction" and continued reductions in total personnel ceilings have combined to produce further attrition in permanent personnel of the Laboratory. Dr. Peter Contacos, an experienced malariologist who headed our Chamblee-Atlanta off-site facility for many years, accepted an administrative position with the malaria research program at the Center for Disease Control. Miss Elizabeth Guinn, an expert microscopist at the Chamblee Unit, retired during the year. Dr. Albert Gelderman accepted a position in another U.S. Public Health Service research facility in West Virginia, and Dr. Andrew Dean of the Pacific Research Unit decided to leave the U.S. Public Health Service and get formal training in public health. We will also lose Dr. Krotoski, an Associate who stayed on for a third year, from the Chamblee Unit, as well as the three Research Associates, Drs. Thomas Hyde, Lee Dobbs, and Stuart Beeber, who are finishing their two year tours in Bethesda.

In the fall of 1971 we were pleased to have Louis Miller, M.D., who was then Associate Professor at Columbia University of New York, join the Laboratory. Dr. Miller now heads the newly reorganized Malaria Section, which includes the malaria research program at Bethesda as well as the facilities in Georgia. Two new Research Associates came on duty in July 1971, Dr. Frederic Knauft in Bethesda and Dr. Timothy Kuberski in Honolulu, and a new Clinical Associate, Dr. Richard Glew came on duty at that time for the Human Malaria Unit in Atlanta.

Several guest workers came to the Laboratory for temporary assignments in 1971. One of these is Dr. Duane Gubler, an entomologist, who has been working at the Pacific Research Section and the other is Dr. Ramero Martinez-Silva, a microbiologist from the Puerto Rico Nuclear Center. Dr. Mohamed El-Sayed Omar continues as a Visiting Fellow with the malaria group at Chamblee, Georgia.

Assistant Laboratory Chief, Dr. Allen Cheever, began a special assignment in July 1971 at NAMRU-3 in Cairo, Egypt for what was expected to be a one year period, but it now appears that he will extend his stay in Egypt for a second year. Dr. Cheever is extending his quantitative work on worm burden and egg concentration in tissues of the human host, which he carried out for Schistosoma mansoni in Brazil, to the S. hematobium species and to a different geographic area of the world. Other foreign travel and assignments by the Staff during the past year included:

Drs. Contacos and Collins to attend a hemisphere-wide meeting on malaria research in El Salvador; Dr. Diamond's visit to India, Israel and Sweden in connection with his PL 480 amebiasis project in India, and

another trip to Mexico to participate in what seems to have developed into an annual affair, an amebiasis symposium; Dr. Pacheco to the Dominican Republic for two weeks to establish contacts and a logistics system for getting filariasis specimens from an urban focus; Dr. Weinbach to Europe (Belgium) to participate in an international symposium; Dr. Neva to Central America for two weeks to complete follow-up studies of his Chagas' disease project; Dr. Collins again to Central America for three weeks for collaborative malaria research with the CAMRS group of El Salvador; Dr. Nash to Brazil for six weeks as a consultant to PAHO on a schistosomiasis field project. Dr. Rosen's travel in the Pacific included visits to Tahiti and New Caledonia, and he also managed to get to Trinidad, Panama, and Costa Rica this year.

During January - April 1972 Dr. Sheffield was in Japan as an invited guest worker at Keio University, School of Medicine, Tokyo.

Dr. Diamond supervised arrangements for visiting guest lecturers during the past year. During Dr. Cheever's stay in Egypt, Dr. Weinbach is serving as Acting Assistant Laboratory Chief.

ADMINISTRATIVE SUMMARY
PACIFIC RESEARCH SECTION
HONOLULU, HAWAII

The research program of the Section continues to be oriented geographically and involves the disciplines of virology, bacteriology, protozoology, helminthology, and entomology. Its main objectives remain, 1) to exploit the advantages for epidemiologic research offered by the relatively simple ecologic environment of Pacific Islands, 2) to investigate diseases which are of particular importance on Pacific island possessions of the United States, and 3) to participate in investigations in Southeast Asia if unique opportunities arise which could contribute to knowledge of diseases which occur in the United States or its territories.

The Section occupied its new facilities at Leahi Hospital at the beginning of the fiscal year but, as might be expected, considerable effort during the year had to be devoted to continuing contacts with various contractors. The entire year was required to straighten out various minor problems.

The Section continues to benefit from having an unusually competent staff - both in professional and supportive categories. The latter personnel (equivalent to 8 full-time positions) are supplied under Contract No. NIH-70-2228 with the Research Corporation of the University of Hawaii (at a current annual funding level of approximately $132,000). A research associate (Dr. Kuberski) was assigned to the Section for the first time at the beginning of the fiscal year and a guest worker (Dr. Gubler) also worked in the Section for eleven months of the fiscal year.

In addition to performing the research work described in the individual project reports, the professional staff of the Section assisted in teaching medical microbiology and epidemiology to students in the University of Hawaii Schools of Medicine and Public Health.

RESEARCH ACCOMPLISHMENTS

DENGUE FEVER

Mosquitoes used as culture medium: In the past isolation and identification of dengue viruses that cause dengue fever has required repeated passage in baby mice or complicated cell culture techniques with considerable problems associated with both methods. Now, an old technique, that of direct intrathoracic inoculation of susceptible mosquitoes with material suspected of containing virus has been found to be a sensitive method to isolate dengue viruses. Furthermore, viral material or antigen can be demonstrated in the infected mosquito by the complement-fixation test. Thus, this procedure provides an improved method for isolating and identifying dengue viruses without need for many special facilities of the usual virus laboratory. Such new methods are especially timely because a variety of fatal dengue fever, previously confined to the Asian mainland and the Philippines, has begun to spread to islands of the South Pacific. Recent outbreaks have occurred in Tahiti and New Caledonia. According to the theory that hemorrhagic dengue can occur wherever the virus is reintroduced at suitable intervals of time, then this severe and sometimes fatal variety of dengue fever might continue to spread to new areas including the Caribbean Islands. (Rosen)

CELLULAR BASIS OF ENERGY TRANSFORMATION IN PARASITES AND MAN

Parasite studies: Non-heme iron is functional in three primary dehydrogenases (glycerol-3-phosphate, succinate, and NADH dehydrogenase) of the respiratory chain of Taenia taeniaeformis mitochondria. DT diaphorase, an enzyme catalyzing NADH oxidation, has been found in cestode mitochondria, and in trophozoites of axenically cultivated Entamoeba histolytica. This enzyme may be of particular physiological significance in parasites with a limited repertoire of oxidative enzymes, since it provides a mechanism for the oxidation of NADH generated during glycolysis.

Mammalian studies: Evidence adduced that non-heme iron participates directly in energy conservation reactions as well as in electron transport of the mammalian respiratory chain. Studies this year indicated that copper may be the active metal in energy conservation in the cytochrome region of the chain.

Human studies: Continued studies with skeletal muscle mitochondria from myopathic patients showed that the most pronounced biochemical abnormality was the low rate of NADH oxidation when respiration was coupled to energy conservation. These findings, in conjunction with histochemical and ultrastructural studies, indicate that mitochondrial defects are associated with certain human myopathies.

Biochemical studies of the respiratory metabolism of human platelets indicated that the basal respiration and a substantial portion of the thrombin-induced burst of oxygen uptake is of mitochondrial origin. Agents known to affect energy metabolism of isolated animal mitochondria can be used effectively to interact with platelet mitochondria in vivo

and thereby aid in elucidating an important mechanism in human physiology.

Biochemical defects in acute, intermittent porphyria, considered to be a hepatic disease, now have been shown to occur in fibroblasts. These findings provide important new evidence that genetic defects not only cause this disease, but that mutations are reflected in the biochemistry of cells other than hepatocytes. (Weinbach)

AMEBIASIS

Viruses in amebae: Studies on the recently discovered viruses of Entamoeba histolytica have been continued at an accelerated pace. Development of a practical assay system has permitted intensive study of their physical and chemical properties. To date, viral agents have been detected in axenic cultures of 7 amebal strains studied. Two morphological types have been distinguished, a polyhedral structure and a filamentous form. Host-range-specifity studies indicate that two, perhaps more, polyhedral agents are involved, while studies with neutralizing antibodies reveal these viruses to be antigenically related. The fact that all of the amebal strains have had origin in patients with active disease from widely separated geographic areas (old and new worlds) points to a possible universality of the amebal viruses, at least in virulent strains of amebae. Whether these or similar viruses are also present in avirulent strains remains to be discovered. Intensive investigations, in animal models, of the possible role amebal viruses may play in amebic disease will be initiated during the coming year. (Diamond; Mattern and Hruska of LVD).

Ameba skin test: Data accumulated in India (PL-480 Agreement #01066-1) on 276 infected and healthy individuals indicates that histolyticin, an antigen prepared from axenically cultivated E. histolytica and extensively used in the serological diagnosis of amebic disease is also useful as a skin-test antigen, especially in extra-intestinal amebiasis. (Diamond; Om Prakash of India- PL-480 Project).

TOXOPLASMOSIS

Life cycle and transmission: The domestic cat has been shown to be a potential reservoir of Toxoplasma gondii. Infection of cats by ingestion of toxoplasma cysts results in the excretion of large numbers of oocysts and production of high antibody titers. Subsequent reinfection results in low oocyst output in some cats or, in most cases, no output. Studies are being conducted to determine the relationship of antibody titer, time and dosage to the probability of reinfection. Such data are important in understanding the role of the cat in the epidemiology of human toxoplasmosis. Ultrastructural studies are also being conducted to further describe the parasite-host relationship during various stages of the life cycle. (Sheffield and Melton)

Immunology and treatment: Diagnosis of toxoplasmosis in pregnant women is of great importance especially in cases where recent exposure is a possibility. Studies are now in progress to determine the significance of low IgM titers during pregnancy. (Lunde)

Earlier work has shown chlorinated lincomycin analogues are effective in treating acute toxoplasmosis in mice. Further studies are being carried out to assess their effectiveness against the cyst stage of the parasite. (Lunde and Powers)

Toxoplasmosis epidemiology: Investigations in the Pacific area during the year continue to provide support for the hypothesis that cats are essential hosts for maintaining toxoplasma in nature and that these animals are usually infected by means of a rodent or avian intermediate host rather than from another cat. Experiments on cockroaches revealed that these insects can harbor and shed infectious oocysts in their digestive tracts for at least three weeks and that their feces can remain infectious for another three weeks. (Wallace of Pacific Research Section)

TRYPANOSOMIASIS

Prevalence of Trypanosoma cruzi infection and and Chagas' disease in Nicaragua: The last follow-up visit to the study areas has been done and a final clinical evaluation of presence and type of heart disease is being made. This will then be correlated with the serologic results to decide how much disease is occurring as a result of infection with the parasite. The few false-positive CF results that occur with certain batches of the tissue culture antigen appear to be due to excessive numbers of beef cells in the antigen preparation. No evidence of serologic cross-reactions to T. cruzi were found in individuals infected with T. rangeli. (Neva)

Analysis of intracellular infection with T. cruzi: The mathematical function that best describes the distribution of trypomastigotes which have invaded host cells is that of a negative binominal distribution. Thus, with a large inoculum, multiple infection of cells is a common event. (Hyde) An interesting but still puzzling "lag period" of about 48 hours exists between the time of cell penetration and the onset of reproduction. The significance of this lag period is not known and it is noteworthy that a similar "lag period" does not occur with T. gondii, another intracellular parasite. (Dvorak) The output of trypomastigotes per infected cell appears to be directly proportional to the number of parasites penetrating the cell, and averages about 500 per organism with the strain of parasite studied. High inputs can result in early host-cell rupture with liberation of amastigote forms of the parasite. (Dvorak) Under certain conditions epimastigotes (crithidia) are able to initiate intracellular infection. (Martinez-Silva)

SCHISTOSOMIASIS

Quantitative study of human schistosomiasis in Egypt: Quantitative study of schistosome infected patients in Egypt shows the severity of disease to be related to the intensity of infection. Infections in these patients are much heavier than those in Nigerian patients, who had less severe disease, suggesting that the principal difference in the disease in these two countries is intensity of infection.

The intensity of schistosome infection can generally be predicted from the concentration of eggs passed in the urine or feces. Under a

variety of experimental conditions, passage of eggs correlated well with the number of eggs being deposited in the tissues.

The above findings suggest that more emphasis should be given to decreasing the intensity of schistosome infection in man, and perhaps less emphasis to complete eradication of the disease, although the latter is a desirable ultimate goal. (Cheever)

Genetic basis for schistosome infection of snails: Studies on the genetics of susceptibility of the snail Biomphalaria glabrata to infection with Schistosoma mansoni have continued with two different Caribbean strains of parasite. At least 8 genetic factors have been found to affect snail susceptibility to the parasite. The susceptibility pattern is simpler in juvenile snails than in adults. Four monogenetic characteristics have been identified in the snail and these are being utilized as markers in an effort to establish linkage groups, i.e., physical characteristics that will indicate susceptibility status. (Richards)

Molluscan parasites that might be useful in biologic control of snails: Microsporidia, continue to be encountered in snails that are being studied, as well as flagellates of the genus Cryptobia. These latter organisms have been found in snails from widely separated geographic areas and some of the flagellates have been observed in intracellular sites. (Richards)

FILARIASIS

Comparative experimental models of filarial infection: The effort which has gone into several different experimental models of filarial infection has begun to pay dividends in terms of a better understanding of dose-response and natural history of infection if immunity is to be studied. For example, relatively more larvae are needed to establish patent infections with the two Brugia species than with other filarial organisms. With all species immunity to reinfection is not complete in terms of development of adult worms as well as production of circulating microfilariae. (Pacheco)

Serologic studies: The Dirofilaria immitis system has shown the most promise for use of metabolic products of microfilariae as antigens to detect specific antibody. The phenomenon of microfilarial (MF) agglutination as an immunologic indicator has been clarified. The subject is confusing because one type of MF agglutination, medusa-head formation, is a non-specific phenomenon that occurs under certain conditions when MF are suspended in plasma from the host. However, immunologically-specific MF agglutination has been shown to be complement dependent and occur in serum or plasma from hosts in which MF do not circulate. (Knauft and Pacheco)

BIOCHEMICAL AND GENETIC STUDIES ON FREE-LIVING AND PARASITIC NEMATODES

Genetic basis for differentiation: Deletion of certain essential nutrients from the growth medium of Caenorhabditis elegans can stop growth and development at any stage. However, many mutants of this nematode have now been obtained which exhibit developmental arrest

specifically in the 3rd to 4th larval stage. In some mutants this arrest is overcome by adding additional nutrients. Other mutants exhibit a selective developmental arrest of certain cell types. Genetic mapping with complementation tests to establish linkage groups is underway with a total of several hundred mutants. (Pertel)

DNA characterization of worms: The DNA extracted from wild-type C. elegans at different stages of the life cycle exhibits variable reassociation kinetics. Thus, the genetic information in somatic tissues of this nematode differs qualitatively from that found in germ line and embryonating tissues. Evidence for selective partial chromosome elimination has also been found. (Pertel and Dobbs)

Sensitive assay for host and parasite acetylcholin-esterases: For studies on the neurotransmitter enzymes of C. elegans a sensitive assay system was developed for acetylcholinesterase which requires material only from single worms or parts of worms. Acetylcholinesterases from male and female Toxocara, Trichuris and hookworm of the dog were studied for their sensitivity to the antihelminthic, dichlorvos. Drug concentrations as low as 10^{-12} M were found to be inhibitory to the parasite cholinesterase. (Pertel and Beeber)

HUMAN MALARIA

Characterization of new isolates of human malaria: Further experience with the relapse pattern of mosquito-induced infections with Central American strains of Plasmodium vivax in human volunteers suggests that the Nicaraguan strain can have earlier and more frequent relapses than other Central American strains of P. vivax. It is noteworthy that the Nicaraguan strain presumably originated from the Atlantic coastal side, whereas the other strains have all come from the Pacific coast side. The frequent relapse pattern of the South Pacific Chesson strain of P. vivax was confirmed after large sporozoite inoculation even though the strain has undergone several years of serial passage in primates. (Contacos, Collins, Krotoski and Howard)

Pathogenesis of the malarial paroxysm: A standardized procedure was developed for estimating parasite density and determining percent of different developmental stages of the parasite at frequent intervals prior to and during the malarial paroxysm. With this approach several key periods in the parasite cycle that relate to the paroxysm have been identified. Whole serum and C4 complement levels are unchanged or rise during paroxysms of the primary attack whereas a transient but significant drop in complement levels is frequently demonstrable during paroxysms of relapses when parasitemia is fairly high. Preliminary attempts to demonstrate a circulating pyrogen in the blood during the early phase of a paroxysm has thus far been unsuccessful. (Neva, Howard, Glew and Miller)

Studies on the organization of the lipid micelle of malaria infected red cells that were started at Columbia University were completed. Changes in the red cell membrane of infected cells are observed by differential binding of an iron colloid when examined by electron microscopy. The membrane changes of the malaria-infected red cell are thought to be an

important factor in pathologic changes resulting from deep vascular schizogony of certain malaria parasites. (Miller)

Coagulation studies during human and simian malaria: Volunteers with human malaria showed no evidence of disseminated intravascular coagulation although various indices of this condition gave abnormal values. (Krotoski, Briesch, Howard)

Even in simian malaria infections which are associated with much higher degree of parasitemia, determination of fibrinogen levels or fibrin-split products gave no convincing evidence of coagulopathy until animals were moribund with P. knowlesi infections. In contrast to a previous report by other workers, heparin had no anti-parasitic effect and did not prolong survival in infected monkeys. (Howard and Krotoski)

MALARIA CHEMOTHERAPY

Lincomycin analogues: Further study of the activity of two lincomycin analogues, U-21 and U-24, against highly resistant Plasmodium falciparum malaria in the owl monkey established the effective dose level for each drug. (Powers and Jacobs) Although these analogues are slowly acting on the blood stages and not practical as a sole agent for treatment of human infections, their effectiveness against P. falciparum suggested their use in combination with a more rapidly acting drug, such as quinine. The U-21 analogue, or Clindamycin, which is commercially available has given encouraging preliminary results vs an RI chloroquine-resistant strain of P. falciparum in man. (Miller, Howard and Glew) Investigation of possible cross-resistance between different varieties of antibiotics in their action against rodent malaria is under study since the antibiotics may become more important in combination drug therapy of human malaria. (Jacobs)

IMMUNOLOGICAL STUDIES OF MALARIA

Fluorescent staining of early EE stages: Preliminary results of detection of sporozoites or early exoerythrocytic (EE) stages of malaria parasites in the tissues by the fluorescent antibody technique have been encouraging. If successful, such a technique would be useful for study of the early phases in development of EE stages. (Krotoski and Collins)

Serologic tests in malaria: In collaboration with the Central American Research Station in El Salvador, periodic serologic surveys for malarial antibody by the immunofluorescence test are being conducted in specific population groups. This information is being correlated with other epidemiologic information to better evaluate presence or absence of malaria transmission. (Collins) Various modifications of the schizont agglutination test are being evaluated. A micromodification, using fixed and preserved antigenic material, has given promising results. (Powers and Miller)

ENTOMOLOGIC STUDIES Mosquito transmission of malaria: Predictable systems for experimental study of the entire life cycle of human malaria parasites, including cyclical transmission through the mosquito, are not yet available. Although many species of simian parasites are transmitted in this Laboratory, factors which influence gametocyte production in the host and their infectivity for the mosquito are poorly understood. After serial blood passage of human strains in the owl monkey ability to infect mosquitoes is lost quite rapidly. One strain of P. vivax has been passaged serially through owl monkeys and mosquitoes but the prepatent period has not shortened and transmission has not been appreciably facilitated. (Collins) Oocyst development in mosquitoes of varying susceptibility has been studied for more precise information on where the block in development occurs in the resistant vector. The effect of antimalarial drugs upon gametocytes is being reevaluated in the light of the oocyst development information. (Omar and Collins)

Serial No. NIAID 120
1. Parasitic Diseases
2. Office of Chief
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Administration, Research Planning and Coordination from the Office of the Chief

Previous Serial Number: Same

Principal Investigator: Franklin A. Neva

Other Investigators: Eugene Weinbach (Acting Assistant Chief)

Cooperating Units: Section on Clinical Parasitology, Laboratory of Clinical Investigation, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	123/12			123/12
Professional	7/12			7/12
Other	116/12			116/12

Project Description:

This project furnishes administrative and supervisory services to research projects in the laboratory as follows:

Research plans are discussed with various investigators and overall research activities are coordinated. Travel of personnel is arranged and secretarial services are provided. Assistance is given in the preparation, editing, and reviewing of manuscripts and reports. Requisitioning and supervision of equipment and supplies and maintenance of the library is provided. Consultation and the liaison activities are maintained with individuals, academic organizations, and other branches of the Federal Government in matters pertaining to the activities of the laboratory. Arrangements are made for supervision of research workers who are in a training status. Centralized facilities and supervision are provided for care of experimental animals used by investigators.

Liaison is maintained with the Section on Clinical Parasitology in the Laboratory of Clinical Investigation with reference to diagnostic services or special investigations that are carried out on patients at the Clinical Center. Dr. Theodore Nash, one of the Clinical Associates from LCI is continuing his research project on schistosomiasis in this Laboratory but maintains certain clinical responsibilities and assignments as a member of LCI.

Administrative reogranization of malaria research activities was effected during the year with all malaria work, both in Bethesda and off-site in Georgia, being placed within a new Section on Malaria. The facilities in Georgia now consist of a Unit on Primate Malaria at Chamblee and a Unit on Human Malaria at the Federal Penitentiary in Atlanta.

Although the Pacific Research Section in Honolulu occupied its new physical facilities at Leahi Hospital in July 1971, various minor problems required continued consultation with contractors for the past year. Through the efforts of Dr. Rosen and Dr. Baron of LVD and a special program of the National Library of Medicine a weekly communications contact via space satellite has been started which facilitates rapid information exchange on scientific and administrative matters between Bethesda and Honolulu.

Mr. Koger continues to supervise facilities and care of experimental animals and technical assistance associated with their use. A similar assignment is handled by Mr. Smith in Chamblee. As property officer, Mr. Duvall oversees our special equipment in the Bethesda Laboratory.

Honors and Awards:

F. A. Neva

Appointed Lecturer in Tropical Public Health at Harvard University School of Public Health, Boston, Massachusetts.

Appointed representative of American Society of Tropical Medicine and Hygiene on newly organized National Council for International Health.

Elected to membership in Association of American Physicians, May, 1972.

Served on Ad Hoc Committee of the National Research Council to evaluate research facilities of Naval Medical Research Institute.

Member of Medical Advisory Board of Leonard Wood Memorial.

Member of Board of Directors of the Gorgas Memorial Institute of Tropical and Preventive Medicine, Inc.

Invited participant to NIH, NICHD sponsored conference on The Biological Role of the IgE system at Vero Beach, Florida, June 4-7, 1971.

Invited Lectures, Conferences, or Talks Given:

1. Harvard School of Public Health on Chagas' disease, Oct. 15, 1971.

2. Discuss a CPC at Boston City Hospital on a patient with fever of unknown etiology, on Oct. 6, 1971.

3. Talk on Chagas' disease studies at School of Public Health and Community Medicine of University of Washington, Seattle, Washington on March 9, 1972.

4. Talk on Malaria at Washington Center Hospitals, Washington, D.C., on April 25, 1972.

5. Talk on Chagas' disease studies and discuss malaria at a clinical conference at Montefiore Hospital, New York on May 22, 1972.

Publications:

Neva, F. A.: Common parasitic diseases of the gastro-intestinal tract. DM. (In Press).

Serial No. NIAID 121-C
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The biology and control of the snail intermediate hosts of schistosomes

Previous Serial Number: Same

Principal Investigator: Charles S. Richards

Other Investigators: None

Cooperating Units: Dr. Donald Heyneman, Hooper Foundation, University of California, San Francisco, California

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To study molluscan genetics with particular attention to applications to control measures. To study prospective agents for biological control of intermediate hosts of the schistosomes. To study the biology of the snails with the aim of improving the efficiency of control measures. To study the contributions and complications of molluscan diseases and molluscan genetics in bio-medical research. To study the causes of tumors in mollusks.

Methods Employed: Selection and crossing experiments are conducted to find additional genetic markers, to characterize linkage groups, to increase knowledge of mechanisms of molluscan genetics, and to investigate further the genetic aspects of molluscan susceptibility to infection and to molluscicides.

Parasites of mollusks are studied to determine: their identity, molluscan host range, mode of transmission, localization and pathology of infection, and interrelations with schistosome infections. Electron microscopy is employed in studies on protozoa and bacteria.

Genetic studies, fresh dissections, and stained serial sections are employed to determine the causes and pathology of tumors in mollusks.

Major Findings: Studies on the genetics of susceptibility of B. glabrata for infection with S. mansoni were continued. Two strains of S. mansoni differing in infectivity characteristics were used; one from Puerto Rico and one from St. Lucia. Both juvenile and adult snails were exposed. Results of testing our laboratory stocks of B. glabrata suggest that at least eight genetic factors affect susceptibility to S. mansoni. Selection has indicated probably seven different susceptibility combinations in B. glabrata.

In crossing experiments with different geographic stocks of B. glabrata, a stock from Dominican Republic has proved relatively incompatible with our laboratory colony of B. glabrata.

Studies on susceptibility of B. glabrata for infection with other trematodes, as compared with S. mansoni, were initiated on an exploratory basis in collaboration with Dr. Donald Heyneman. Echinostoma paraensei proved infective to all stocks of B. glabrata exposed to it, including stocks refractory to all strains of S. mansoni tested.

It was demonstrated that an abnormal growth character in B. glabrata, including swollen tentacles and everted preputium, is inherited as a simple recessive. The everted preputium is nonfunctional, so that these snails can self-fertilize, but in cross fertilization can serve as females only. This is the fourth visible monogenetic character we have found in B. glabrata, and it has been established that the four are on different chromosomes. These serve as markers for four linkage groups: I, basic pigmentation (wild type, blackeye, or albino); II, pearl formation; III, antler tentacle; IV, swollen tentacles and everted preputium. Data so far suggest that the monogenetic adult insusceptibility factor is probably on a fifth chromosome.

A collection of Biomphalaria straminea, an intermediate host for S. mansoni in some localities, was obtained from Brazil. This species was found to have a monogenetic pigment factor with three alleles as in B. glabrata; wild type, blackeye, and albino. A population of snails in a lake in Baton Rouge, Louisiana, identified as "Tropicorbis havanensis" was studied by several investigators in the 1940's. This was considered the only snail species "endemic" to the U. S. susceptible to S. mansoni infection. Snails from our laboratory colony descended from a snail collected in Baton Rouge in 1948, probably the last of that snail population, have been mated with B. straminea from Brazil with production of fertile hybrids. This suggests the Baton Rouge snail colony was probably a temporary exotic population accidentally introduced from South America.

Studies on tumors in B. glabrata, some genetic and some not, were continued.

Studies on protozoan and bacterial parasites of snails were continued. It was shown that severe pathology due to the microsporidian Coccospora brachynema, Richards and Sheffield 1970, was the result of an inherited host-parasite relation in B. glabrata.

Flagellates of the genus Cryptobia were found in the seminal receptacle of B. straminea. Three species of Cryptobia, related to the trypanosomes, were compared from the following snails: Gyraulus and Segmentina from Taiwan, Drepanotrema from Puerto Rico, and B. straminea from Brazil. Intracellular stages of these flagellates have been observed in fresh preparations, and electron microscopy has revealed interesting ultrastructural features.

Significance to Bio-medical Research and the Program of the Institute:

Establishing stocks of B. glabrata, of various combinations of juvenile and adult susceptibility to strains of S. mansoni differing in infectivity, provides material for bio-chemical, biological and cytogenetic intraspecific analyses of the basic mechanisms of susceptibility.

Need for evaluation of the role of genetics in snail control continues. Refractory B. glabrata may prove useful in biological control of schistosomiasis by dilution or replacement of populations of susceptible snails in endemic areas. Progress in understanding causes of susceptibility or insusceptibility of B. glabrata to infection with S. mansoni accelerates the prospect of safe field testing of refractory snail stocks for control. Development of a refractory stock of B. glabrata incompatible with the endemic snail population in a problem area may avoid dilution of the introduced insusceptibility by interbreeding.

Establishing markers for some of the various linkage groups in B. glabrata increases our capacity to analyze invisible and complex genetic characters such as host-parasite relations.

Discovery of the monogenetic three allele pigment character in B. straminea provides a genetic marker enabling studies of the genetics of susceptibility in that snail species for comparison with B. glabrata.

Information on a variety of tumors observed in B. glabrata, many of which are genetic, should help in analyses of tumors of mollusks collected in the field.

Microsporidia, amebae, and bacteria cause extensive damage in some snail hosts. These and other snail diseases need to be examined for potential human infection, and prospective use as agents in biological control of snails. It is essential to be aware of the occurrence of these infections and their relations with the snails and other parasites to plan, conduct, and interpret experimental research on snails with validity.

Results to date increasingly implicate genetics in host-parasite relations involving various parasites.

Proposed Course of the Project: Genetic studies on B. glabrata will be continued to: (a) seek additional genetic markers; (b) expand our knowledge of linkage groups; (c) characterize and establish true breeding stocks of the various susceptibility combinations of B. glabrata for S. mansoni infections; (d) test additional stocks of B. glabrata and strains of S. mansoni as available and (e) continue studies of incompatibility between certain stocks of B. glabrata.

Strains of S. mansoni exhibiting different infectivity characteristics will be crossed to study the genetic aspects of infectivity.

Genetics of susceptibility in other snail species, such as B. straminea, will be compared with B. glabrata.

Studies on protozoan and bacterial infections in mollusks will be continued: including relations to the molluscan hosts, effects on other parasites, potential for human infection, and as a variable factor in experimental research. Electron microscopy studies on an intracellular bacterial parasite of Bulinus jousseaumi, an intermediate host of Schistosoma haematobium, will be continued in collaboration with Dr. Roger M. Cole (LM, project NIAID-65).

Molluscan tumors will be studied by fresh dissections and culturing, serial sections, genetic selection and crossing, and environmental manipulation to determine causal factors.

Publications:

Richards, C. S. and Sheffield, H. G.: Unique host relations and ultrastructure of a new microsporidian of the genus Coccospora infecting Biomphalaria glabrata. Proc. IVth International Colloquium on Insect Pathology with the Soc. for Invertebrate Pathology. 439-452, 1970.

Richards, C. S.: Urceolaria viridis n. sp., a ciliate (Peritrichida, Mobilina) with elongate symbiotic green algae. J. Protozool. 18: 410-413, 1971.

Richards, C. S.: Biomphalaria glabrata genetics: pearl formation. J. Invertebrate Pathology (In Press).

Richards, C. S. and Merritt, J. W., Jr.: Genetic factors in the susceptibility of juvenile Biomphalaria glabrata to Schistosoma mansoni infection. Am. J. Trop. Med. Hyg. (In Press).

Serial No. NIAID 121-H
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Indivisual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Pathogenesis of Schistosome Infections in Mammalian Hosts.

Previous Serial Number: Same

Principal Investigator: Allen W. Cheever
(Detail assignment U.S. Naval Medical Research Unit #3, Cairo, Egypt - 7/1/71 - 6/30/72)

Other Investigator: Kendall G. Powers

Cooperating Units: Department of Medical Zoology, Walter Reed Army Institute of Research (Drs. Sadun and Erickson); Department of Pathology, Peter Bent Brigham Hospital, Boston, Mass. (Drs. von Lichtenberg, and Smith); Department of Parasitology, Southwest Foundation, San Antonio, Texas (Drs. Kuntz and Myers); East Africa Institute for Medical Research (Dr. Eyakuze); Departments of Pathology, Cairo University Medical School (Drs. Elwi and Kamel) and Ein Shams University (Dr. Soliman), Cairo, Egypt; Naval Medical Research Unit No. 3, Cairo, Egypt; Tanta Mission Hospital, Tanta, Egypt; Department of Urology, Cairo University Medical School (Dr. Torky); Department of Pathology, U. Bahia, Brazil (Dr. Andrade)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	13/12			13/12
Professional	11/12			11/12
Other	2/12			2/12

Project Description:

Objectives: The interactions between various mammalian hosts and schistosomes of medical importance are studied, particularly the dynamics of egg production by the parasite and the excretion or destruction of eggs by the

host. Pathologic lesions are studied in relation to the above variables and to physiologic changes, such as portal hypertension and hepatic and gastrointestinal function. Infected persons are studied using quantitative techniques to determine the role of intensity of infection in producing clinically significant disease, to evaluate the differences in disease seen in different geographic areas and to provide baseline data against which the relevance of animal models can be measured.

Methods Employed: Laboratory rodents and primates infected with S. mansoni, S. haematobium or S. japonicum are studied during the course of infection and are sacrificed at various intervals after infection. Pathological and parasitological findings at autopsy are correlated with observations before death. Surgical specimens from schistosome infected patients are studied to correlate pathological changes with the numbers of eggs present in the tissue. Autopsy cases are examined to relate the presence and intensity of schistosome infection to the lesions noted.

Major Findings: The intensity of S. haematobium infection in man is clearly related to the severity of lesions seen, principally hydroureter and hydronephrosis. As in experimental animals, egg concentration in the ureters is about as heavy as that in the bladder, suggesting that primary involvement of the ureter is clinically important. The median intensity of infection is 5 or 10 times higher than that reported from Nigeria, where severe urogenital lesions are infrequent.

Active S. haematobium infections, with adult worms and live eggs present in the tissues, are not frequently seen at autopsy. Sufficient cases have been seen to indicate that the basic tissue reaction is similar in most respects to that in experimentally infected primates. Also noteworthy is the focal nature of the disease, i.e., worms remain in a given location for extended periods of time, resulting in massive focal egg deposition and focal lesions in the genitourinary and gastrointestinal tract.

Chronic S. haematobium infections in man, in contrast to those in experimental animals, result in massive accumulation of calcified eggs in the tissues. This is seen in children as well as in adults. In about half of the nearly 100 infected cases studied, more than 50% of the eggs recovered are outside the genitourinary system, mainly in the lunds and colon.

S. mansoni infected patients in Egypt show the same hepatic pathology as those studied in Brazil. Comparison of 100 wedge liver biopsies from patients with portal hypertension show the same basic pathology as that seen in similar material from Brazil, and autopsy specimens are also comparable. Intestinal polyposis seen in autopsy and surgical specimens from patients with S. mansoni and S. haematobium infection is entirely different thatn the colonic pathology seen in Brazilian material.

Proliferative epithelial lesions of the urinary bladder seen in infected persons show basic differences from those produced by schistosome

infection in experimental animals. Most bladder cancers in infected patients are squamous, as expected, rather than transitional cell, as in the experimental model. However, the lack of transitional cell proliferation in bladders with squamous carcinoma or in severe schistosome infection without neoplasia is surprising. The numbers of S. haematobium eggs passed in the urine preoperatively has generally correlated well with the number of worms recovered from the surgically excised bladders.

S. japonicum infected monkeys and baboons lost relatively large amounts of protein in their stools and showed severe, segmental lesions of the small intestine and colon at autopsy. Liver lesions were marked in capuchin monkeys, but pathology differed considerably from that seen in man. The development of immunity in monkeys was much greater following heavy than following light infection. Marked inhibition of oviposition by worms was seen in immune animals. The number of eggs passed in the feces remained proportional to the number of eggs laid by the worms.

Grivet monkeys repeatedly exposed to S. mansoni showed relatively little resistance to the development of adult worms from the re-exposing doses, but the monkeys did partially inhibit oviposition by the worms.

Significance to Biomedical Research and the Program of the Institute:

The quantitative study of schistosome infection in man allows more meaningful comparison of the disease in different geographic areas, in this instance S. haematobium infection in Egypt appears to produce greater disease than that in Nigeria because of the greater intensity of infection seen here, and S. mansoni infections in Brazil and Egypt seem basically similar, except for the presence of colonic polyposis in Egypt. The findings suggest that most of the severe lesions of schistosomiasis can be prevented by lowering the intensity of infection. Eradication of schistosome infection from individual patients or geographic areas, although desirable, is thus felt not to be crucial to the health of the individual.

The passage of eggs in the excreta of infected patients and experimental animals has, under the most varied of circumstances, proved to be a reasonably accurate means of estimating the rate at which eggs are being deposited in the tissues.

Proposed Course of the Project: Studies of infected patients will continue along the lines indicated above, with an intensive effort to include more active cases with S. haematobium infection. Emphasis will also be placed on the study of renal lesions in S. haematobium infection. The relation of tryptophane metabolism and bladder cancer in primates infected with S. haematobium will be investigated.

Honors and Awards:

Dr. Cheever served on the Council of the American Society of Tropical Medicine and Hygiene and was invited to present a paper to the Middle East Medical Assembly.

Publications:

Andrade, Z. A. and Cheever, A. W.: Alterations of the intrahepatic vasculature in hepatosplenic schistosomiasis mansoni. Am. J. Trop. Med. Hyg. 20: 425-431, 1971.

Erickson, D. G., Lucia, H. L., von Lichtenberg, F., Cheever, A. W., and Sadun, E. H.: Schistosoma haematobium infections in five species of primates. Exp. Parasitol. 29: 126-137, 1971.

Kuntz, R. E., Myers, B. J., and Cheever, A. W.: Schistosoma haematobium infection in the opossum (Didelphis marsupialis): involvement of the urogenital system. Bull. Wld. Hlth. Organ. 45: 21-26, 1971.

Lee, H. G., Cheever, A. W., and Fairweather, W. R.: Influence of parasite strain on chemotherapy of murine infections with Schistosoma mansoni. Bull. Wld. Hlth. Organ. 45: 147-155, 1971.

von Lichtenberg, F., Sadun, E. H., Cheever, A. W., Erickson, D. G., Johnson, A. J., and Boyce, H. W.: Experimental infection with S. japonicum in chimpanzees. Parasitologic, clinical, serological and pathologic observations. Am. J. Trop. Med. Hyg. 20: 850-893, 1971.

Kuntz, R. E., Cheever, A. W., and Myers, B. J.: Proliferative epithelial lesions of the urinary bladder of monkeys infected with Schistosoma haematobium. J. Nat. Cancer Inst. 48: 223-235, 1972.

Anderson, L. A. and Cheever, A. W.: Comparison of geographic strains of Schistosoma mansoni in the mouse. Bull. Wld. Hlth. Organ. (In press).

Powers, K. G. and Cheever, A. W.: Comparison of geographic strains of Schistosoma mansoni in the rhesus monkey. Bull. Wld. Hlth. Organ. (In press)

Cheever, A. W. and Powers, K. G.: Schistosoma mansoni infection in rhesus monkeys: comparison of the course of heavy and light infections. Bull. Wld. Hlth. Organ. (In press).

Sadun, E.H., Erickson, D.G., Hickman, R.L., von Lichtenberg, F., and Cheever, A.W.: Experimental Infections with Schistosoma mansoni and S. haematobium in the Chimpanzee. S. Gaafar (Ed): Pathology of Parasitic Diseases. Purdue University Studies, Lafayette, Indiana, 1971, pp. 105-120.

Serial No. NIAID 121-I
1. Parasitic Diseases
2. Host-Parasite Relations
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Serologic Studies on Filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigator: Anastasia Stanley

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana. Dr. Angel M. Ponce, San Pedro de Macoris, Dominican Republic. Dr. Martin S. Wolfe, Department of State, Washington, D. C.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	17/12			17/12
Professional	15/12			15/12
Other	2/12			2/12

Project Description:

Objectives: To study serologic changes in experimental and natural infections with various filariae in man and animals. Antigens from different stages in the life cycle of homologous or heterologous parasites are used in these studies.

Methods Employed: Indirect tanned-cell hemagglutination (IHA) is used as the basic technic to evaluate humoral antibody response. Diffusion in agar and immunoelectrophoresis are used as adjuncts in analysis of antigens; fluorescent antibody technics are used to demonstrate antigen-antibody complexes with living nematodes.

Sera used in the above tests are from: (a) jirds, dogs, cats or Patas monkeys infected once or periodically with Brugia malayi, (b) jirds, dogs or cats infected once or periodically with B. pahangi, (c) dogs experimentally infected with Dirofilaria immitis, (d) Patas monkeys infected with

D. corynodes, (e) sera from U. S. citizens returning to the United States who are suspected or proved to have infections with filariae - Onchocerca volvulus, Loa loa, Dipetalonema perstans - and (f) sera from families in San Pedro de Macoris, Dominican Republic, in which one or more members have microfilaremia of Wuchereria bancrofti.

Antigens used for serologic tests are: (a) fractionated extracts of adult male and female D. immitis, (b) metabolic products of third-stage infective larvae of Dipetalonema viteae, B. malayi or B. pahangi and (c) metabolic products of microfilariae of D. immitis, B. malayi or B. pahangi.

Major Findings: IHA with extracts of adult D. immitis was used to provide a preliminary assessment of antibody in all of the sera processed. With sera from people in an endemic area - San Pedro de Macoris - antibody was detected in low titer, or not at all, if the individual had microfilaremia. By contrast, sera from people without microfilaremia, but with high eosinophilia or history of clinical filariasis, had high antibody titers. Antibody was rarely detected in sera from people treated by the State Department, except for some infected with O. volvulus or L. loa. The latter had high titers of antibody.

Antibody was first detected in sera from Patas monkeys two weeks after infection with B. malayi. Peak antibody titers were noted at three weeks. These persisted eight to twelve weeks and then decreased. Decrease in antibody titer coincided with appearance of microfilaremia and by the time the latter was well established, antibody was no longer detectable. It is noteworthy that with metabolic products of third-stage infective larvae as antigen, antibody was detected in sera from one of these monkeys only, and only after reinfection.

Evaluation of sera from jirds experimentally infected or reinfected with B. malayi or B. pahangi with extracts of adult D. immitis indicates that antibodies are detected early in the infection - two weeks - and persist at high titer - 1:1,280 to 1:2,560 - well past the time that microfilaremia is stable. This is in contrast to results with sera from Patas monkeys in which antibody is undetectable after patency. Preliminary tests with metabolic products of third-stage larvae indicate that antibodies are not detectable in sera from jirds given a single injection of larvae, but they are present in sera from reinfected jirds. Thus, as in sera from Patas monkeys, anti-larval antibody may indicate active reinfection.

In early work with third-stage larvae of D. viteae it was noted that erythrocytes were not sensitized by stored metabolic products. To achieve sensitization of cells, it was necessary to mix living larvae with the erythrocytes. In contrast to this, metabolic products of the Brugia spp. larvae can be stored in a refrigerator (5°C), at least a few days, or in a freezer (-20° C) at least one week. This provides antigens that can be more readily quantitated and minimizes the necessity for daily supplies of larvae from mosquitoes.

Studies on the use of metabolic products of microfilariae as antigens indicate that those of D. immitis can be used to detect antibody in dogs in which microfilariae do not circulate. In that this antibody cannot be detected with metabolic products of microfilariae of Brugia spp., the antibody appears specific.

Significance to Bio-medical Research and the Program of the Institute:

Serologic tests used as aids in the diagnosis of filariasis lack specificity, although some are quite sensitive. Adequate evaluation of currently available tests has proved difficult because of lack of sera from controlled infections, as well as ignorance of changes that may occur in levels of antibody with respect to time. These difficulties have proved particularly troublesome in trying to evaluate results of tests done with sera from patients suspected to have nonpatent infections - clinical symptomatology without microfilaremia. Availability of sera from controlled infections with various species of filariae in various hosts provides material with which to approach some of the questions raised. Similarly, availability of sera from people in an area in which Bancroftian filariasis is endemic can provide an assessment of changes in antibody with respect to time.

Antibodies detectable with extracts of adult D. immitis appear to be nonspecific, but as these extracts are actually a mixture of substances, it may be that some of these could provide "group specific" antigens as was originally hoped. By contrast, metabolic products of third-stage infective larvae appear to be quite specific. Anti-larval antibodies appear to be associated with reinfection and therefore may be useful to determine infection of people in an endemic area whether or not microfilaremia is detectable. Similarly, anti-microfilarial antibodies may be useful to detect "occult" infections or disease associated with microfilariae.

Proposed Course of the Project: While extracts of adult D. immitis will continue to be used to provide an assessment of antibody in sera from people or animals experimentally infected, emphasis will be on establishing test systems with metabolic products of third-stage larvae and of microfilariae. It is hoped that microfilariae of W. bancrofti can be obtained from people in San Pedro de Macoris, Dominican Republic, to begin to evaluate sera from people in an endemic area with homologous parasites.

Serial No. NIAID 121-J
1. Parasitic Diseases
2. Host-Parasite Relationship
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host-parasite relationships in filariasis

Previous Serial Number: Same

Principal Investigator: Guillermo Pacheco

Other Investigators: Dr. Stuart Beeber, Research Associate, PHS
Dr. Frederic Knauft, Research Associate, PHS

Cooperating Units: Dr. Thomas C. Orihel, Delta Regional Primate Center, Covington, Louisiana; Dr. George S. Tulloch, San Antonio, Texas; Dr. Angel M. Ponce, San Pedro de Macoris, Dominican Republic.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	38/12			38/12
Professional	28/12			28/12
Other	10/12			10/12

Project Description:

Objectives: To study the interaction between filariae and their vertebrate hosts. Additionally, other nematodes living in tissue would be studied. These studies are directed to provide a better understanding of filariasis and other nonpatent helminthiases in man.

Methods Employed: Brugia malayi, B. pahangi, Dipetalonema viteae, Dirofilaria immitis and D. corynodes, as well as appropriate arthropod vectors, are maintained for experimental study. Blood and tissues from monkeys infected with D. corynodes or B. malayi, people infected with W. bancrofti and adult Toxocara canis are received from collaborating laboratories.

To study the effects of various stages of filarial life cycle, as

well as possible interaction of these stages, vertebrates are given : (1) third-stage larvae from an appropriate arthropod vector, (2) adult worms by subcutaneous or intravascular surgical implantation or (3) washed microfilariae by intravenous infusion. Numbers of leucocytes, eosinophils and, when present, microfilariae are estimated as preset intervals. When vertebrate hosts are necropsied the adult worms that are recovered, as well as representative samples of tissue, are preserved for later study.

Mice and rabbits are given eggs of T. canis by gavage and, for some experiments, mice are injected intravenously with hatched larvae. Tissues of mice are recovered at various time intervals after infection to determine whether antibody or antigen can be detected by fluorescent technics.

Major Findings:

A. Infections with filariae. To determine basic parameters of host and parasite response to infection with B. malayi, groups of jirds were given single injections of varying numbers of larvae, fortnightly injections of preset numbers of larvae or fortnightly injections of preset numbers of larvae starting after microfilaremia was present. Microfilaremia was not detected in jirds given a single injection of less than 50 larvae, but was detected in all others regardless of the number of larvae given or whether the jirds were reinfected. The average prepatent period for all groups was 20 weeks with a range from 14 to 26 weeks. Length of prepatency was not significantly modified by different schedules of infection or reinfection. Rate of growth, as evidenced by weight gain, was unaffected in jirds given a single injection of larvae, but in reinfected jirds was impressively less than in uninfected jirds. Maximum eosinophilia was seen in all groups of jirds six weeks after the first injection of larvae. Thereafter eosinophilia decreased, regardless of reinfection, and was indistinguishable from that of uninfected jirds by the 20th week. Infection of jirds with B. pahangi has the same features noted above for B. malayi, except that the prepatent period ranges from 10 to 16 weeks. These studies are still in progress.

B. Mode of action of diethylcarbamazine. Work in Japan with Litomosoides carinii indicated that the immunological status of a host determined whether or not microfilariae would be cleared following treatment with diethylcarbamazine. To determine whether the same phenomena are operative with other filariae, dogs with D. immitis infection, microfilariae only, adult worms and microfilariae, but without experience of larval stages or with experience of larval stages plus microfilariae, but no adult worms will be treated with a standard course of diethylcarbamazine. Antibody titers will be monitored throughout the experiment and the condition of the host and of the worms will be determined at necropsy.

C. Occult filariasis. Microfilariae have been repeatedly infused intravenously into dogs. Host response to these infusions has been monitored by following the course of microfilaremia and by observing the interaction between living homologous or heterologous microfilariae and plasma from the treated dogs. Microfilaremia disappeared after several injections. Thereafter

microfilariae did not circulate regardless of the number injected. Microfilariae were unaffected by plasma from the dogs before the injections were started or while microfilaremia was present. However, they were promptly agglutinated by plasma from dogs in which microfilariae no longer circulated. Agglutination of microfilariae was noted in untreated serum or plasma, but not in serum or plasma heated at 56°C for 30 minutes. Addition of guinea pig serum to heated dog serum or plasma restored agglutination.

D. Visceral Larva Migrans. Attempting to detect antigen (S) of T. canis in tissues of infected mice, gamma globulin from rabbits infected with the same parasite was labelled with fluorescein and then placed on frozen sections of mice infected with T. canis. Fluorescence was not detected in any of the sections tested to date.

Significance to Bio-medical Research and the Program of the Institute:

Basic interaction of host and filariae are poorly understood. The availability of the jird as a model in which two of the filariae that parasitize man will develop, provides an opportunity to learn more about these interactions. Additionally, as Brugia spp. also parasitize cats, dogs and monkeys, a comparison of the interaction of these parasites with their respective hosts should result in an understanding of basic changes associated with these parasites.

Although diethylcarbamazine has been a major drug in the management of bancroftian filariasis, its mode of action is unknown. It is hoped that the studies currently in progress will provide more rational approaches that will eventually lead to full understanding of this problem.

Agglutination of microfilariae in sera from patients with clinical filariasis without microfilaremia has been extensively documented, but confused with "Medusa Head" formation in sera from people with microfilaremia. These two phenomena have been clearly separated. The former has been shown to be an immunologic reaction while the latter is a distinctly separable occurrence apparently associated with sheathed microfilariae only.

Proposed Course of the Project: Continuing studies on filariasis will emphasize the effects of reinfection in various hosts with human filariae. Special attention will be given to establishment of models in which occult filariasis can be studied. In collaboration with Dr. Bloch of Western Reserve University exploratory trials to determine whether microfilariae can be visualized in lung or liver in vivo will be attempted.

Honors and Awards:

Dr. Pacheco was an invited participant at the U.S.-Japan Cooperative Medical Science Program, Parasitic Disease Conference, July 26 and 27, 1971 and Workshop on Development of Filariae in Mosquitoes, March 20 and 21, 1972.

An invited participant and co-editor of the Proceedings of the Second University of Florida Symposium on Canine Heartworm Disease, October 1972.

An invited participant in the National Conference on Research Animals in Medicine.

Dr. Pacheco gave lectures in Parasitology to first-year medical students at the University of Pittsburgh School of Medicine.

Continued participation in meetings of the Tropical Medicine and Parasitology Study Section, DRG, NIH.

Publications:

Pacheco, G., Atkins, J. and Gurian, J. 1972. Quantification of infection of ticks with Dipetalonema viteae. J. Parasitol. (In Press).

Serial No. NIAID 122-G
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Entamoeba histolytica and other parasitic protozoa

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Carl F. T. Mattern and Bruce P. Phillips

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	39/12			39/12
Professional	8/12			8/12
Other	31/12			31/12

Project Description:

Objectives: To develop and refine techniques for the axenic cultivation of Entamoeba histolytica, related amebae and other parasitic protozoa; to define their in vitro nutritional requirements, to study their metabolism, and mechanisms of pathogenicity. To ascertain factors involved in cyst production of Entamoeba. To study naturally occurring viruses of E. histolytica. To develop techniques for cryopreservation of parasitic protozoa, to study mechanisms involved in resistance of these organisms to freezing injury.

Methods Employed: Entamoebae or other parasitic protozoa freed of naturally associated microorganisms by use of microisolation procedures and/or a variety of antibiotics are established in monoxenic cultures with single species of trypanosomatids or bacteria, then adapted to grow under axenic conditions. In vitro growth is monitored through use of a Coulter electronic counter and/or standard counting chambers.

Axenically cultivated E. invadens is exposed to crithidial lysates and the process of the ensuing encystment of the amebae studied by electron microscopy.

Viruses of E. histolytica are characterized and studied using classical virological techniques.

Protozoa are suspended in a variety of crypopreservatives (glycerol, dimethylsulfoxide, glucose, etc.) and the effects of varying freezing and thawing rates on recovery of viable cells cooled to temperature of liquid nitrogen (-196°C) are studied.

Laboratory animals are inoculated via laparotomy with amebae cultivated under a variety of conditions and pathogenesis is studied.

Major Findings: During the past year the main research effort has been directed toward a detailed examination of the viruses recently discovered in Entamoeba histolytica. Viral agents have been detected in axenic cultures of 7 amebal strains studied. Each strain has been found to be resistant to its indigenous virus. On the other hand, the viruses from each strain will infect and lyse the amebae of one or more of the other 6 strains.

The intracellular development of the viruses has been studied with the electron microscope. Two morphological types, both of which may occur in the same amebal strain, have been distinguished: a polyhedral structure and a filamentous form. Clearly identifiable polyhedral virus is seen only in the cytoplasm of infected cells especially in the perinuclear area, whereas the filamentous form is observed first in the nucleus and then in the cytoplasm.

Cross-infection studies to determine host-range specificity within the several amebal strains indicate that two or more polyhedral viruses are involved, while studies with neutralizing antibodies reveal a kinship between these viruses.

Attempts to produce cytopathology in chick and mouse embryo cells and human fibroblast cultures with these agents have failed, as have attempts to produce pathology in suckling and adult mice inoculated by a variety of routes.

Development of a practical assay system has permitted investigation of the physical and chemical properties of these viruses. To-date, the polyhedral agent (HB-301)→ HK-9 has been intensively studies with the following results: 1) Thermal stability. In amebal growth-medium the virus is inactivated approximately one log when subjected to -20°C. Repeated freezing and thawing results in additional loss of titer but to a lesser extent. Inactivation is more rapid at -78.5°C. At 4°C the virus is stable for at least 20 days. At 37°C it is inactivated in medium at the rate of 0.8 log per 24 hrs, while in Tris buffer (pH 7.6) inactivation is more rapid. 2) pH stability.

The virus is relatively stable only within the pH range of 6.5 to 8.0. 3) Effect of selected reagents. Inactivated by ethyl ether, chloroform, EDTA (10^{-2M}), sodium dodecyl sulfate (1%), Alrowet detergent (0.1%), and partially by desoxycholate (1%), Non Idet detergent (1%), and Tween 20 (0.1%). Unaffected by trypsin (0.1%), Tris buffer (0.1 M, pH 7.6), hylauronidase (100y/ml), Non Idet (0.1%) and phospholipase. 4) The virus is tightly bound to membranes which form upon lysis of the amebae and all efforts to free the virus using a variety of physical and chemical means have failed.

Lack of a virus-free amebal strain has hampered the study of the ameba-virus system. For this reason, attempts have been made to free at least one strain of its indigenous virus. Cultures of normal HB-301 amebae contain 10 x $^{2.5}$ ID_{50} virus as detected by rupturing 5 x 10^6 amebae and assaying with HK-9 amebae, a strain particularly sensitive to this viral agent. On the other hand, virus cannot be detected in HB-301 amebae grown in the presence of HB-301 virus antiserum. Considering the possibility that prolonged exposure to antiserum could result in freeing the ameba of its virus, the amebae were grown with antiserum for 35 consecutive subcultures. Despite this treatment virus was again detectable at the original titer after 4 to 6 subcultures in the absence of antiserum. Although this work has not produced the desired results, the observations reported suggest the existence of a lysogenic system.

Continuing efforts to develop a bank of axenic cultures of representative species of the Genus Entamoeba have resulted in axenization of 1) an ameba from human feces which is morphologically similar to E. histolytica and E. histolytica-like amebae (Laredo type) but physiologically different in that its optimal growth temperature is 33 C. 2) a non-cyst forming ameba, probably E. barreti, from the snapping turtle, Chelydra serpentina.

Significance to Bio-medical Research and the Program of the Institute:

The relation of the amebal viruses to amebic disease is as yet unknown and requires serious investigation especially in light of the role of virus in bacterial disease. The finding of virus in 7 amebal strains having origin in seriously ill patients widely separated geographically (old and new worlds) points to a possible universality of these viruses at least in virulent amebal strains. Whether these or similar viruses are also present in avirulent strains remains to be discovered.

Knowledge to be gained from study of the viruses of a primitive animal eukaryote should add to our insight regarding the overall understanding of viral-mammalian cell interactions.

Proposed Course of the Project: In collaboration with staff members of the LVD and LMI special emphasis will be placed on a search for viruses in avirulent strains of E. histolytica. Search will also be made for virus in other species of Entamoeba as well as various other pathogenic protozoa.

Attempts will be made to alter the course of amebic disease in animal models through treatment with amebal viruses. Patients with amebic disease are being screened serologically for antibodies against amebal viruses. Continued efforts will be made to further characterize the viruses of amebae and attempts to cultivate these agents in vertebrate cell cultures will be intensified. An electron microscopic study of encystation in the snake pathogen, E. invadens, is in progress. Efforts to develop techniques for the axenic cultivation of other parasitic protozoa will continue, as will efforts to improve the techniques of cryopreservation of these organisms.

Honors and Awards:

Chairman, American Type Culture Collection Advisory Committee to the Collection of Protozoa.

Member, Board of Trustees, American Type Culture Collection.

Member, WHO Expert Advisory Panel on Parasitic Diseases (General Parasitology).

Member, Executive Committee, Society of Protozoologists.

Member, Editorial Board, Cryobiology.

Invited Conference Leader, Fourth Seminar on Amebiasis, sponsored by the National University of Mexico and the Mexican Institute of Social Security, Mexico City, February 23-25, 1972.

Publications:

Gelderman, A. H., Keister, D. B., Bartgis, I. L. and Diamond, L. S. 1971. Characterization of the deoxyribonucleic acid of representative strains of Entamoeba histolytica, E. histolytica-like amebae, and E. moshkovskii. J. Parasitol. 57: 906-911.

Gelderman, A. H., Bartgis, I. L., Keister, D. B. and Diamond, L. S. 1971. A comparison of genome sizes and thermal-denaturation-derived base composition of DNAs from several members of Entamoeba (histolytica group). J. Parasitol. 57:912-916.

Diamond, L. S. and Bartgis, I. L. 1971. Axenic cultures for in vitro testing of drugs against Entamoeba histolytica. Arch. Invest. Med., Sup. 1, pp. 339-348.

Diamond, L. S., Mattern, C. F. T., and Bartgis, I. L. 1972. Viruses of Entamoeba histolytica. I. Identification of Transmissible virus-like agents. J. Virol. 9: 326-341.

Mattern, C. F. T., Diamond, L. S. and Daniel, W. A. 1972. Viruses of Entamoeba histolytica. II. Morphogenesis of the polyhedral particle ($ABRM_2$→HK-9)→HB-301 and the filamentous agent ($ABRM_2$)→HK 9. J. Virol. 9: 342-358.

Phillips, B. P., Diamond, L. S., Bartgis, I. L. and Stuppler, S. A. 1972. Results of intracecal inoculation of germfree and conventional guinea pigs and germfree rats with axenically cultivated Entamoeba histolytica. J. Protozool. (In Press).

Serial No. NIAID 122-J
1. Parasitic Diseases
2. Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Basis for Growth and Differentiation of Parasitic and Free-Living Nematodes

Previous Serial Number: Same

Principal Investigator: Dr. Ruth Pertel

Other Investigators: Dr. Leland G. Dobbs and Dr. Stuart E. Beeber, Research Associates

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	44/12			44/12
Professional	32/12			32/12
Other	12/12			12/12

Project Description:

Objectives: To investigate the generalized life cycle of parasitic and free-living nematodes utilizing techniques from biochemistry, genetics, and neurobiology. Specifically to study DNA synthesis and DNA-DNA reassociation kinetics during the life cycle of Caenorhabditis elegans var. Bristol; to further define the genetics and population dynamics of various mutants. To investigate sensory modulation in the wild type of C. elegans and in its mutants at various stages of differentiation and development in an attempt to elucidate the sensory mechanism involved in the transitional phase of nematodes when a free-living mode is exchanged for a parasitic mode and the development of the reproductive system begins.

Methods Employed: Free-living Caenorhabditis elegans var. Bristol cultured as synchronous populations under axenic conditions (free of all living associates) are used as the initial test system. Chemotactic, motility,

drug resistant, and developmental mutants are used to study the neuronal basis for the responses of nematodes to their environment. Developmental mutants are studied in regard to their genetics, population dynamics, and media effects upon growth and development.

Nucleic acid methods: Nematodes grown axenically in synchronous culture are harvested and disrupted in a French Pressure Cell. DNA obtained from the homogenate is then characterized by thermal stability. Reassociation kinetics are determined optically with a recording spectrophotometer and by hydroxyapatite elution for rapidly reassociating fractions. DNA content of the worms at different stages of their life cycle are determined by a microfluorescence method.

Acetylcholinesterase assay: A radioactive micromethod depending upon column elution of the product of hydrolysis is employed.

Chemotactic assay: A biphasic (semisolid agar and distilled water system) is used to determine the sensitivities of various mutants to chemicals.

Major Findings:

1. Dichlorvos, mechanism of action on host and parasite specific and nonspecific acetylcholinesterases.

Dog intestine and brain were assayed and the properties of their enzymes studied. The parasitic nematodes, Toxocara, Trichuris and hookworm from the same dog were studied in the same fashion. Since the microassay system can be utilized to study enzyme homogenates from single worms or even parts of worms the size of Trichuris, it was possible to study the enzymes of the males and females of parasites as well as the anterior and posterior portions. Dichlorvos was found to be inhibitory to the acetylcholinesterases at levels as low as 10^{-12}M in the parasites. Inhibition of the host intestinal enzyme was of the same order of magnitude. The antihelminthic behaved as an irreversible inhibitor of acetylcholinesterase. The assay system was initially developed for studies on the neurotrasmitter enzymes of C. elegans (Pertel and Beeber).

2. DNA characterization of wild-type Caenorhabditis elegans:

DNA isolated from synchronous cultures at different stages of the life cycle of C. elegans exhibits different reassociation kinetics. This information coupled with previous information from radioautography and ultracentrifugation studies indicates that genetic information contained in the somatic tissues of nematodes differs qualitatively and quantitatively from that found in germ line and embryonating tissue. Selective partial chromosome elimination during embryonic development has been previously described in Ascaris (Boveri, 1887). We have found evidence of this same phenomenon in C. elegans which indicates that somatic tissue does not possess the same amount of genetic information as germ line cells (Pertel and Dobbs).

3. Genetics: Complementation tests have been worked out and six linkage groups established. Genetic mapping of 300 mutants is underway.

The holding stage (dauerlarva) of parasitic nematodes prior to infection of the host usually occurs at some point prior to the development of the gonads. In the Strongyles and in C. elegans (a putative evolutionary relative of the Strongyles), development of the gonads begins at the third to fourth stage molt transition. We are interested in the degree that genetic vs. environmental information affects this transition. Deletion from the growth medium of hemin and steroid, essential nutrients of C. elegans, can produce environmentally determined holding stages, i.e. growth stoppage with maintenance of that stage of growth at a particular molt stage and even halt reproduction in the wild type adult. However, in approximately 30 mutants, mapped as separate genes, the third to fourth molt stage is the one uniformly affected in terms of growth and developmental phenomena (Pertel).

4. Dauermodification mutant: A mutant was described which retains its second stage cuticle and which manifests a life cycle dependency upon a change of medium after it has gone through a reproductive cycle and growth to the third stage. Behavioural differences were also evident in pre-third stage larvae which were no longer apparent in the adult. Specific media components which would reinitiate growth at this stage were determined (Dobbs and Pertel).

5. Neurosensory modulation: Sensory mutants of C. elegans were further defined in regards to their chemotactic responses. Description of the sensory apparatus of the worms, papillae, amphids, etc. and their synaptic connections and cytochemical neurotransmitters was initiated (Pertel).

Significance to Bio-medical Research and the Program of the Institute:

Parasitic nematode infections of man, domestic animals, and plants are protean in character and world-wide in distribution. While there exist many anti-helminthic drugs and preparations, little is known about the exact mechanism of action of these compounds, especially when the state of knowledge is compared with what is known about antimicrobial agents. In our pursuit of basic knowledge about the developmental and neurological processes of the nematode we developed an assay for one of the enzymes involved in neurotransmission, acetylcholinesterase. This assay system became useful in the study of the specific mechanism of action of Dichlorvos, once considered as a broad spectrum antihelminthic for man.

The nematode system also offers a good study system for the understanding of the basic biology of differentiation and development. It is an in vivo system that uniquely separate the differentiation of one tissue, the gonad, from other fully differentiated tissues. Most other systems where blast stages undergo differentiation in a well-controlled fashion are those in tissue cultures, i.e., in vitro systems which frequently become contaminated with transforming viruses, type of blast cell is difficult to characterize, genetic information is complicated by gene dosage and loss effects when chromosomes undergo ploidy changes, etc. The information derived from the

nematode studies will also provide understanding of the basic interactions attendant upon the development of parasitic nematodes as influenced by the host which so often occurs at the third to fourth molt stage transition when the gonad develops.

Proposed Course of the Project:

The environmental stimuli required to initiate the development of the gonads and the sensory modulation of this information will be pursued by further studies with a variety of sensory and developmental mutants. The mapping of the developing nervous system of the nematode will be attempted at the light and electron microscope level. In addition, the neurotrasmitter characteristics of junctions in the anterior end of the worm will be attempted.

In situ hybridization of the various DNAs will be performed to locate the chromosomal or cytoplasmic origin of the varous satellite and rededundant DNAs. The function of specific DNAs eliminated at the various stages of development will be pursued.

Honors and Awards:

An invited lecture was presented by Ruth Pertel at the Medical Research Council Laboratory of Cellular and Molecular Biology, Cambridge, England, November 1971. She also tutored medical students at Jesus College, Cambridge University.

Dr. Pertel was a member of a site visit team for a Program Project of NICHD to SUNY at Buffalo, November 1971.

An invited speaker on June 21 and 22, 1972 to the Laboratory of Biochemical Pharmacology, NIAMD, Bethesda, Maryland.

Serial No. NIAID 123-B
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathological physiology and histochemistry of parasitic diseases

Previous Serial Number: Same

Principal Investigator: Teresa I. Mercado

Other Investigators: None

Cooperating Units: Laboratory of Microbiology, NIAID (NIAID-207)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To gain a better understanding by means of physiological and histochemical studies of the pathological physiology of parasitic diseases.

To elucidate factors which may complicate a parasitic infection and consequently modify the known pathological physiology of the host induced by the infection.

Methods Employed: Assays of calcium, respiration, and oxidative phosphorylation are made on mitochondria from livers of mice infected with the Tulahuen strain of Trypanosoma cruzi employing atomic absorption spectroscopy, Warburg respirometry, Biuret, Kjeldahl, and standard histochemical techniques.

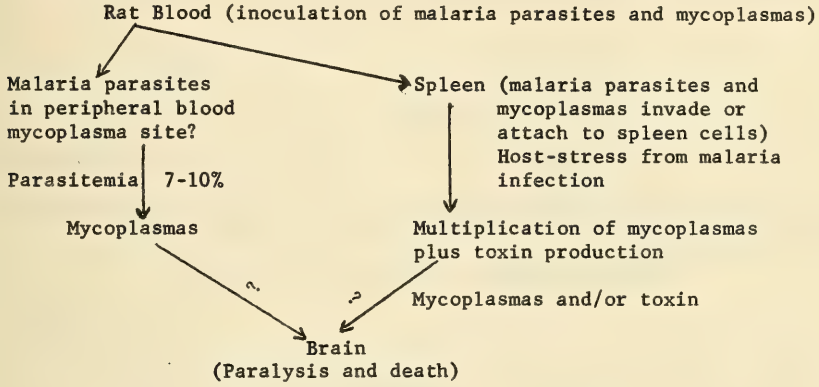
The role of mycoplasma in the paralyzing syndrome of rats infected with Plasmodium berghei is studied by means of blood-passaging in splenectomized rats, animal inoculations, active and passive immunization, and using standard culture techniques.

Parasitemia levels are determined by hemocytometer counts and microscopic examination of blood smears stained with Giemsa.

Major Findings: In studies completed last year it was observed that livers from mice infected with the Tulahuen strain of Trypanosoma cruzi had a significantly higher content of calcium than livers of normal mice. The enhancement observed, however, was considerably lower than that reported by other investigators following poisoning with an organic chemical, such as carbon tetrachloride, in which pathologic changes such as liver glycogen depletion, lipid infiltration, and necrosis, comparable to those exhibited by the T. cruzi-infected livers, also occurred. The observed difference in calcium accumulation between these pathologic conditions suggested the possible impairment of mitochondrial function in the T. cruzi-infected livers. Calcium accumulation is a process involving energy utilization in which mitochondria play a predominant role. Initially, it was pertinent to determine whether mitochondria of infected livers contained more calcium than corresponding controls. The results indicate that in infections of 7 to 8 days and parasitemias ranging from 10 to 72 million trypanosomes per ml of blood, an increase of 55 percent in calcium content occurred over the normal level. This finding is in essential agreement with that reported earlier, based on assays of liver homogenates, and demonstrates that the organelles have the capacity to sequester the greater part of the excess calcium produced as a result of the parasitic infection. Accompanying analyses employing the chelating agent, ethylene glycol tetracetic acid (EGTA), revealed that the calcium accumulation was not related to an in vitro effect resulting from homogenization but apparently resulted from sequestration of the cation in vivo. These findings suggested further examination of the calcium-regulating function in terms of the mitochondrial calcium-binding properties. Measurement of maximal calcium uptake under in vitro optimal conditions disclosed a higher uptake by organelles from infected livers but the question whether or not the rates of influx and efflux is modified, an important factor to consider in assessing the significance of the observed higher uptake, remains to be examined. The occurrence of marked necrosis in T. cruzi-infected livers and the observed reduction in the activity of the mitochondrial enzymes, succinate dehydrogenase and cytochrome oxidase, reported earlier, suggested a study of mitochondrial respiration and oxidative phosphorylation. Based on biochemical assays completed so far these functions appear to be essentially normal, judging from the phosphate-oxygen ratios obtained in the presence of succinate or glutamate. The only abnormal change observed was a considerably higher respiration, with succinate, of mitochondria from infected livers.

In previous attempts employing indirect immunofluorescence and hemaglutination techniques we had not succeeded in detecting the presence of antibody in rats exhibiting, or after recovery from, the Plasmodium berghei paralyzing syndrome. This year, with the use of the metabolic inhibition test for the detection of mycoplasma antibody it was possible to detect a significant increase in antibody titer against Mycoplasma neurolyticum, the organism suspected to be the cause of the syndrome, fourteen days after challenge, in rats which had survived a paralytic attack. Rats receiving the same challenge but without evidence of paralytic disease exhibited low antibody titers to this Mycoplasma species. Active immunization employing toxin and whole cells

of M. neurolyticum reduced the incidence of paralysis and death by 36 to 48 percent in rats inoculated with the P. berghei paralyzing strain. A combination of active and passive immunization provided also only partial inhibition to the paralytic disease. A longer period of passive immunization may have provided more effective protection. Based on the indirect evidence presented in previous reports and in the present one, the following sequence of events is suggested in the production of the paralyzing syndrome.


Rat Blood (inoculation of malaria parasites and mycoplasmas)
Malaria parasites in peripheral blood mycoplasma site?
Spleen (malaria parasites and mycoplasmas invade or attach to spleen cells) Host-stress from malaria infection
Parasitemia 7-10%
Mycoplasmas
Multiplication of mycoplasmas plus toxin production
Mycoplasmas and/or toxin
Brain
(Paralysis and death)

Significance to Bio-medical Research and the Program of the Institute:

Basic cellular studies provide a means for understanding the physiological mechanisms involved in the response of the host to parasitic infections and may lead to the identification of specific parasite substances involved in pathogenesis. Histochemical studies aid in localizing the altered sites within tissues. Physiological studies quantitate the alteration of activity observed histochemically and aid in defining further the physiological mechanisms that may be damaged.

The paralytic syndrome resulting from the association between a malarial infection and a mycoplasma indicates the increasing importance of research related to the behavior of mixed infections. The observed role of the spleen in the production of the paralyzing syndrome indicates the need for continued attempts to define further the role of this organ in the malaria-mycoplasma interaction.

Proposed Course of the Project: Biochemical and cytochemical studies of mitochondria from T. cruzi-infected liver and heart tissues will be continued. Studies on calcium regulation by the organelles will be extended employing radioisotope techniques.

Attempts to culture the paralyzing agent will be continued. Further attempts to define the role of the spleen in the paralyzing syndrome will be made.

Honors and Awards: None

Publications:

Tully, J. G. and Mercado, T. I. Mixed Infections: The interaction of mycoplasmas and malaria parasites. In Pathogenic Mycoplasmas. Ciba Foundation Symposium. Elsevier, Amsterdam. In Press.

Serial No. NIAID 123-C
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biochemical Mechanisms of Energy Metabolism in Mammalian and Parasitic Organisms.

Previous Serial Number: Same

Principal Investigator: Eugene C. Weinbach

Other Investigators: James Dvorak, and Louis Diamond

Cooperating Units: Medical Neurology Branch, NINDB (King Engel, Pieter Kark)
Metabolic Diseases Branch, NIAMD (Sidney Wolfe, Joseph Muenzer)
Metabolism Branch, NCI (Donald Tschudy)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	30/12			30/12
Professional	15/12			15/12
Other	15/12			15/12

Project Description:

Objectives: To conduct fundamental studies on the mechanism of aerobic energy metabolism in mammalian and in parasitic organisms. Specifically: (A) To continue studies of electron transport and oxidative phosphorylation in mammalian cells and mitochondria, and (B) comparative studies with cells and mitochondria from parasites.

Methods Employed: Animal tissues are fragmented by mechanical or enzymatic disruption of the cells and mitochondrial fractions are isolated by differential centrifugation. Mitochondria also are isolated from human biopsy specimens. Similar procedures are used to isolate mitochondria from cestodes, the parasites principally used in current studies.

Entameba histolytica, strain HK-9, is cultivated axenically in Diamond's liquid medium, and the active trophozoites collected from 72-hour cultures by centrifugation.

Human platelets are isolated from freshly drawn whole blood by differential centrifugation.

Fibroblasts are grown from skin punch biopsies obtained from normal patients and from patients with acute, intermittent porphyria. The cells are harvested by trypsinization and centrifugation.

Cellular and mitochondrial constituents are determined and characterized by appropriate enzymatic, chemical and physical methods. Manometric and polarographic techniques are used to measure rates of oxidation.

Major Findings:

A. Parasite Studies

1. Cestode mitochondria: Studies were continued on the role of non-heme iron in the respiration of cestode mitochondria. It now has been established that non-heme iron proteins are associated with three primary dehydrogenases of the respiratory chain of Taenia taeniaeformis. Polarographic determinations, and low temperature electron spin resonance studies, done in collaboration with Dr. H. Kon, NIAMD, have demonstrated that non-heme iron is functional in electron transport catalyzed by glycerol-3-phosphate dehydrogenase, succinate dehydrogenase, and NADH dehydrogenase. Of particular interest was the finding that an active succinate dehydrogenase is present in T. taeniaeformis mitochondria in view of the absence of any significant succinate oxidase activity in these organelles.

Two mechanisms have been eluciated for the oxidation of NADH in cestode mitochondria. (1) NADH is oxidized via the respiratory chain by means of NADH oxidoreductase. This enzyme is sensitive to known inhibitors of electron transport in mammalian mitochondria, and contains non-heme iron. (2) NADH is oxidized via DT diaphorase. This enzyme is not sensitive to inhibitors of electron transport and does not contain non-heme iron. In contrast to the mammalian enzyme, DT diaphorase in cestodes to be localized largely in the mitochondria instead of the cytosol.

2. Entameba histolytica: Investigation of the respiratory metabolism of intact trophozoites of axenically grown E. histolytica were continued in collaboration with Dr. L. Diamond. Evidence was adduced during the year that this parasite contains an active DT diaphorase (see above). There is no indication that NADH is oxidized via the respiratory chain in E. histolytica. Vitamin K_3 (Menadione) is the most effective electron acceptor. In its sensitivity to inhibitors, the protozoan enzyme resembles the menadione reductase of bacteria more closely than it does the mammalian enzyme. DT diaphorase may be of particular physiological significance in a parasite such as E. histolytica, because of its limited repertoire of oxidative enzymes.

Trophozoites of E. histolytica exhibit a decline in respiratory activity during storage. This was correlated with a loss of sulfhydryl compounds from the parasite and deterioration of the cellular membrane. Treatment of the freshly harvested parasite with detergents also abolished respiratory activity. These observations indicate that structural integrity of the membrane is required for optimal aerobic metabolism of the ameba.

B. Mammalian studies.

Continued studies on the role of metals in energy-conservation reactions of the mammalian respiratory chain provided new evidence that non-heme iron components are functional in these reactions. In addition to localization of non-heme iron at Site 1 (NADH to cytochrome b span of the respiratory chain) we have shown that energy coupling at Site 2 (cytochrome b to c) is uncoupled by iron-chelators. Our most significant finding this year is that copper may be the active metal in energy conservation at Site 3 (cytochrome c to oxygen). Experiments with reagents having high affinity for copper disclosed that these reagents are effective uncouplers of oxidative phosphorylation. Although copper is known to participate in electron transport in the cytochrome region of the respiratory chain, this is the first evidence that this metal may be involved directly in energy conservation reactions.

C. Human Studies:

1. Biochemical changes in skeletal muscle mitochondria from myopathic patients: The collaborative study with Drs. K. Engel and P. Kark, NINDS, designed to determine whether mitochondrial abnormalties provide a biochemical basis for certain myopathies, was continued. This year, mitochondria were isolated from five patients diagnosed as having ragged red fiber myopathy. Mitochondria isolated from clinically normal muscles served as controls. Confirming our preliminary results reported last year, the most pronounced biochemical abnormality found was the low rates of oxidation with exogenous NADH when respiration proceeded via the mitochondrial pathway leading to energy conservation, but not when the extramitochondrial non-energy yielding pathway was utilized. Mitochondria from the myopathic patients oxidized all substrates tested at normalized rates slightly below the mean rates found with mitochondria from control patients. Correlative experiments utilizing a micro slice technique and radiochemical substrates also disclosed that muscle samples from the myopathic patients were impaired in their respiratory metabolism. These findings, in conjunction with histochemical and ultrastructural studies, now strongly indicate that mitochondrial defects are associated with certain human myopathies.

2. Respiration of human platelets: This study, initiated last year in conjunction with Dr. S. Wolfe and Mr. J. Muenzer (NIAMD), was continued with emphasis on assessing the role of mitochondria in platelet respiration. Selected inhibitors of mitochondrial respiration consistently caused greater inhibition of the basal (endogenous) platelet respiration than that of the thrombin-induced burst of oxygen uptake. Cyanide (0.2mM) caused 100% inhibition of the basal rate, but only 15% inhibition of the

thrombin-induced oxygen burst. Similar differential effects were observed with oligomycin, antimycin and rotenone. Primary inhibitors of platelet function (Prostagladin, theophylline, dibutyrl cyclic AMP, aspirin) only inhibited the basal rate by 20%, but caused significantly greater inhibition (30 to 100%) of the thrombin-induced oxygen burst. These findings, indicate that the basal respiration and a substantial portion of the thrombin-induced oxygen burst of human platelets is of mitochondrial origin.

3. Biochemical studies of fibroblasts from patients with acute, intermittent porphyria: This study, initiated last year, was continued in collaboration with Dr. D. Tschudy, NCI. Biochemical defects in acute, intermittent porphyria, considered to be a hepatic disease, now has been shown to occur in fibroblasts. Our studies demonstrated that enzymes involved in electron transport and in porphrin biosynthesis are altered in fibroblasts of porphyric patients as compared to normal fibroblasts. Biochemical abnormalites associated with this disease lead to accumulation of porphyrins. The normal level of these compounds is believed to be modulated by the mitochondrial ratio of NAD/NADH. We found that NADH oxidation in fibroblasts from porphyric patients was less sensitive to rotenone and amytal (specific inhibitors of mitochondrial NADH oxidoreductase) when compared to normal fibroblasts. Correlative studies showed that uroporphyrinogen synthase, a key enzyme in porphyrin biosynthesis, was decreased in fibroblasts from porphyric patients. These studies provide important new evidence that genetic defects not only cause this disease but that the mutations are refected in the biochemistry of cells other than hepatocytes.

Significance to Biomedical Research and to the Program of the Institute:

This investigation, because of its fundamental nature in attempting to eluciate vital biochemical mechanisms common to all aerobic cells, has obvious importance for biochemical research in general. This is shown in the several collaborative projects related to clinical studies. This investigation also provides a basis for understanding bioenergetic problems associated with parasitism. Studies of energy metabolism in parasites, apart from their intrinsic and comparative interest, contribute toward a more rational understanding of the host-parasite relationship, and may provide information for chemotherapeutic exploitation.

Proposed Course of the Project: The parasite studies will be continued with emphasis on mechanisms of energy conservation reactions in cestodes. Studies of the aerobic metabolism of Entameba histolytica will be extended to other species of pathogenic ameba, and to further delineation of the enzymatic pathways involved in the respiration of these putative anaerobes.

The mammalian studies will be pursued in regard to the role of metals in oxidative phosphorylation. The collaborative projects involving biochemical studies of human skeletal muscle and fibroblasts will be continued. The study of respiratory metabolism of human platelets has been concluded.

Honors and Awards:

E. C. Weinbach was an invited participant in an international symposium on the Comparative Biochemistry of Parasites sponsored by the Janssen Pharmaceutica Company, Beerse, Belgium, September 1 - 3.

E. C. Weinbach was appointed to Ad Hoc Committee by the Gurgeon General, USPH, to advise on certain chemical and biochemical problems.

Publications:

Hackenbrock, C. R., Rehn, T. G., Weinbach, E. C., and Lemasters, J. J.: Oxidative phosphorylation and ultrastructural transformation in mitochondria in the intact ascites tumor cell. J. Cell Biol. 51: 123-137, 1971.

Hackenbrock, C. R., Rehn, T. G., Gamble, Jr., J. L., Weinbach, E. C., and Lemasters, J. J.: Ultrastructual transformation in the mitochondrion and the energy state of the cell. In Proceedings of Colloquim on Bioenergetics: Energy Transduction in Respiration and Photosynthesis. (Quagliariello, E., Papa, S. and Rossi, C. S. editors) Adriatica, Editrice, Bari, Italy, 1971, p. 285.

Weinbach, E. C.: Role of non-heme iron in cestode reapiration. In Comparative Biochemistry of Parasites (Van den Bossche, H., editor) Academic Press, New York and London, 1972, p. 433.

Kark, R. A. P., Weinbach, E. C., and Engel, W. K.: Oxidative metabolism in small samples of normal and diseased muscle. Proceedings of the 2nd International Congress on Muscle Diseases, Perth, Australia, Excerta Medica (Kakulas, B., editor) 1971-1972, (in press).

Serial No. NIAID 123-E
1. Parasitic Diseases
2. Physiology and Biochemistry
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biophysical Parasitology

Previous Serial Number: Same

Principal Investigator: James A. Dvorak

Other Investigators: Thomas P. Hyde and Ramiro Martinez-Silva (Guest Worker)

Cooperating Units: Biomedical Engineering and Instrumentation Branch, Division of Research Services, NIH

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	42/12		12/12	54/12
Professional	24/12		12/12	36/12
Other	18/12			18/12

Project Description:

Objectives: To utilize biophysical techniques for the study of parasitological problems of medical importance. Major emphasis has been directed toward a better understanding of the host-parasite interactions in Chagas' disease. The approach is two-fold: (1) to obtain a clear understanding of the interaction at the cellular and subcellular level of Trypanosoma cruzi with vertebrate cells and (2) to relate this information to what is observed in a population of infected cells in vitro and, hopefully, the general course of events in vivo.

Methods Employed:

(1) In vitro cultivation

The controlled-environment culture system technique which fulfills the exacting requirements for quantitative and qualitative analysis of cellular and subcellular events has been combined with a newly developed technique which allows the analysis of large populations of cells under highly controlled

physiological conditions and rigidly reproducible geometries. This combination of techniques permits the correlation of phenomena occurring at the individual host cell-parasite level to extended populations of host-parasite interactions.

(2) Quantitative microscopy

The use of cinemicrography has been extended from time-lapse (time-scale compression) to real time and high speed (time-scale expansion). This has permitted a closer analysis of several phases of host cell-parasite interactions which occur with non-linear time bases.

The use of interference microscopy has been extended to an analysis of events occurring during the early stages of intracellular existence of protozoan parasites. This form of analysis is possible as a result of the recently developed semiautomated data acquisition and analysis system for the interference microscope which permits discrete (non-integrative) measurements to be made.

(3) Ultrastructural studies

In collaboration with Dr. L. Miller, ultrastructural studies of the charge distribution on the surface of parasites have been initiated. The technique involves the exposure of parasites to monodisperse colloidal iron in the presence of various counter ions. The electron dense colloidal iron which attaches to the parasite is then visualized in the electron microscope.

Major Findings:

(1) The interaction of Trypanosoma cruzi with vertebrate host cells

A continuation of the study of the interaction of the Ernestina strain of T. cruzi with primary bovine embryo muscle cells has confirmed the usefulness of this model in the elucidation of events occurring during the intracellular cycle. The division of the cycle into major phases has permitted analysis of specific events in the cycle which are obscured or, at best, modulated by subsequent events. Primary emphasis has been placed on: (a) quantitation of the penetration phase, (b) analysis of the "reorganization" phase, (c) elucidation of the mechanism resulting in a "lag period" between "reorganization" and the onset of reproduction and (d) quantitation of parasite output as a function of input.

(a) The rate of penetration of host cells by T. cruzi can be determined with a standard error of less than 10% of the mean values. This penetration rate quantitatively describes the penetration of host cells as a function of several defined parameters. An analysis of penetration as a function of host-cell type revealed that the mean number of trypomastigotes which penetrate a cell is directly proportional to the mean area of the projected host-cell profile. This correlation has been established from analysis of two cell populations (HeLa and primary bovine embryo muscle) which differ significantly

with respect to both morphology and physiology.

A quantitative analysis of trypomastigotes penetrating host-cells has been found to fit a negative binomial distribution. These data represent a quantitative description of the phenomenon of multiple penetration of individual host-cells observed in the controlled-environment culture chamber.

Proof that this technique is applicable to a quantitative analysis of the penetration phase of organisms other than T. cruzi was shown by its successful employment in experiments with Toxoplasma gondii. Contrary to reports that the distribution of T. gondii penetrating host cells is Poisson in nature, this parasite also exhibits a negative binomial distribution among host cells.

(b) The "reorganization" phase of the interaction of T. cruzi with vertebrate cells consists of the gradual transition from trypomastigote to amastigote. Analysis by interference microscopy indicates that the dramatic phase change which occurs during "reorganization" is due to a change in mass per unit volume of the parasite and not a change in the absolute dry mass of the parasite per se. Physiologically, this could represent an accommodation of the parasite to an intracellular existence. The apparent disappearance of the parasite when observed by phase contrast microscopy is due to the non-linearity of the phase-amplitude relationship in this instrument.

(c) The "lag period" between "reorganization" and the onset of reproduction of the Ernestina strain of T. cruzi in primary bovine embryo muscle cells has been timed in single host-parasite interactions at 53.5±7.6 hours. This "lag period" may represent either the elapsed time prior to the synchrony of the parasite to some phase in the host cell's cycle, the time required for the parasite to bring some aspect of the host cell's physiology under parasite control or the time required for the physiology of the parasite to be shifted from an extracellular, highly motile flagellate to an intracellular reproductive form. Experiments are in progress in an attempt to understand the underlying mechanisms of the "lag period." It is noteworthy that a "lag period" does not exist with T. gondii infections. The significant difference in the status of the intracellular parasite (i.e., T. cruzi is free in the host cell's cytoplasm; T. gondii is host cell plasmalemma-bound) may be a morphologic indication of marked physiologic differences in the intracellular stage of these two parasites.

(d) A cell-by-cell analysis of the entire intracellular cycle of the Ernestina strain of T. cruzi in bovine embryo muscle cells indicates that the number of parasites produced in the host cell is almost directly proportional to the number of parasites penetrating the cell. The input of one trypomastigote results in an output of nearly 500 trypomastigotes (i.e., about 8 to 9 generations). Within reasonable limits, higher input numbers result in proportionally higher output numbers. These data seem to indicate that the reproductive phase of the parasite may be under genetic control. It is, however, modulated by host cell physiology. Extremely high inputs result in early host cell death and the liberation of immature parasites. The

state of parasite maturity, in this case, appears to be dependent upon the time of cell death.

(2) Ultrastructural Studies

Preliminary data indicate that localized areas of high net negative charge may exist on the surface of T. cruzi trypomastigotes. Attempts are being made to correlate this data with in vitro observations of the penetration of T. cruzi into vertebrate cells. The intracellular amastigote stage of T. cruzi appears to have little if any net negative surface charge.

(3) Infection of Mammalian Cells by the Epimastigote Stage of T. cruzi.

Preliminary data from a study of the interaction of T. cruzi epimastigotes with vertebrate cells reveal that this developmental stage is capable of initiating an infection. The mechanism by which the parasite gains entry into the host cell and the outcome of the infection remain to be determined.

Significance to Biomedical Research and the Program of the Institute:

(1) The study of host-parasite interactions at the cellular and subcellular level is a topic of critical importance. The outcome of this interaction directly influences the physiologic state and survival potential of both the host and the parasite.

Our ability to subdivide the entire interaction into analyzable units has significantly improved the probability of finding biological "pressure points" for attempted interdiction of the parasite's cycle. For example, interdiction of the penetration phase of the interaction will totally interupt the natural life cycle of the parasite. Our data indicate that T. cruzi which appears to have a prediliction for certain target organs in nature is equally capable of penetrating any cell type it is exposed to. Thus, the prediliction of the parasite for certain target organs does not reflect the success or failure of penetration of the cells of other organs but rather the inability of the parasite to continue its natural cycle in these cells.

The applicability of this approach to the study of other human infectious diseases has been demonstrated by our ability to utilize our techniques in an analysis of the interaction of T. gondii with vertebrate cells. The wealth of comparative information which is being accumulated should be of significant value in the understanding of parasitic diseases in general.

(2) The methodology and research philosophy being developed are directly applicable to other areas of biomedical research. For example, the controlled-environment culture system developed with this program is now in routine use in laboratories with such diverse interests as oncology, immunology, neurophysiology and morphogenesis.

Proposed Course of the Project: Research will continue in an attempt to further our understanding of the interaction of T. cruzi with vertebrate host cells in vitro and relate this information to the course of Chagas' disease in nature. Basic information obtained with the controlled-environment culture system will be utilized in more complex in vitro models. Fully differentiated vertebrate myocardial cells will be used in an attempt to determine the effects of infection with T. cruzi on this cell type as well as the genesis of the focal myocardial lesions seen in nature.

Attempts are being made to further expand the approach to include an analysis of immunologic parameters in the interaction.

Population studies have reached a point where it now appears to be feasible to begin studying the effect of parameters such as parasite strain, pH and temperature on host cell penetration.

Honors and Awards:

An invited lecture was presented by J. A. Dvorak at NIAID-LCI Medical Grand Rounds on June 9, 1971 entitled: "The design and application of a controlled-environment cell culture system for quantitative light microscopy."

An invited lecture was presented by J. A. Dvorak at the annual dinner meeting of the New York Microscopical Society on November 3, 1971 entitled: "A new approach to the study of human parasitic disease via quantitative light microscopy with notes on its applicability to other research areas."

A patent for the controlled-environment culture chamber developed in the Laboratory of Parasitic Diseases was filed in April 1972 by Browdy and Neimark (patent agents) representing the U. S. Government with J. A. Dvorak listed as the principal inventor.

Publications:

Dvorak, J. A., and Stotler, W. F.: A controlled-environment culture system for high resolution light microscopy. Exp. Cell Res. 68: 144-148, 1971.

Dvorak, J. A., Clem, T. R., and Stotler, W. F.: The design and construction of a computer-compatible system to measure and record optical retardation with a polarizing or interfere microscope. Jour. of Microscopy. (In press).

Dvorak, J. A., and Schmunis, G. A.: The interaction of Trypanosoma cruzi with mouse peritoneal macrophages." Exp. Parasit. (In press).

Serial No. NIAID 125-A
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Toxoplasmosis

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: Marjorie L. Melton

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	15/12			15/12
Professional	15/12			15/12
Other				

Project Description:

Objectives: The study of Toxoplasma gondii infections in cats to compare with the course of the disease in man; to determine the stages and sites of development of the parasite in the cat; to investigate the possibility of other animals serving as natural hosts; to determine possible mechanisms by which oocyst infections can be acquired by man and animals.

Methods Employed: Cats are infected by feeding them cysts in mouse brain or oocysts isolated from feces of infected cats. Oocysts are collected by zinc sulfate or sucrose flotation. Infectivity of concentrated oocysts is determined by feeding them to mice and subsequent checking for organisms or anti-toxoplasma antibodies. Tissues from infected animals are used for histolotical or ultrastructural studies. Cultured cells are used for in vitro studies of development of sporozoites which have been artificially excysted from oocysts and inoculated into the cultures.

Major Findings: Cultivation of endogenous forms of toxoplasma has been attempted using various cell lines. Monolayers of canine kidney, feline kidney embryonic bovine kidney, rat embryo, embryonic rat lung and kidney, vero cells, human embryonic lung and bovine embryonic intestine have been inoculated with either excysted sporozoites or cyst organisms. Coverslips examined at intervals between 4 hours and 6 days after infection contained infected cells. However, all organisms have been observed dividing by endodyogeny. No evidence of schizogony or gametogony has been seen.

Study of growth of toxoplasma in organ cultures has also been started. Results similar to those seen in monolayer cultures have been obtained. As yet no other forms of division than endodyogeny have been seen.

When appropriate animals have been available reinfection with toxoplasma has been attempted. In general, cats have not become reinfected regardless of the interval since the primary infection if they have dye test titers above 1:64. Several infections have resulted when cyst organisms were used for challenge in cats having titers of 1:32 or less. For comparative purposes immunity resulting from primary exposure to Isospora felis is being studied. There is no apparent cross immunity between T. gondii and I. felis. Successful reinfection was obtained with I. felis four months after the primary infection.

Attempts have been made to determine the role of the extraintestinal migration of toxoplasma after oral infection with cyst organisms. Pyrimethamine and sulfadiazine were administered to cats for 10 days. Two days after initiation of therapy cyst organisms were fed. Three of four cats passed oocysts beginning on days 14-17 after infection. One cat was negative. The control cat passed oocysts as would be expected on day 4. This drug regimen did not prevent infection but apparently suppressed development until the drug levels subsided. Experiments are in progress to determine where the drug acted and at what point in the toxoplasma life cycle.

Studies of Isospora canis have been initiated to determine the response of this organism when introduced into abnormal hosts. Although toxoplasma infects numerous animals it is not known whether other coccidia can go through at least a portion of their life cycle in an abnormal host. Oocysts of I. canis have been fed to mice. After 4 days the mice were killed and impression smears made of mesenteric lymph nodes. Although rare, organisms were seen in some of the smears.

Significance to Bio-medical Research and the Program of the Institute:

The identification of the cat as the natural host for T. gondii provides a new insight into the epidemiology of toxoplasmosis and could explain the wide-spread existence of anti-toxoplasma antibodies in man and animals. In addition, the linking of T. gondii, whose classification has been uncertain for many years, to the coccidia provides the investigator with many model systems which could be used for comparison purposes in the study of toxoplasmosis in normal and abnormal hosts. The cat's role as a

reservoir animal may be significant in the cosmopolitan distribution of the disease. Information on the immunological aspects of the disease in cats would show the possibility of reinfection and may provide new leads relative to the cat being an important reservoir of the organism. Elucidation of the various stages of the life cycle in the cat would contribute to a better understanding of the disease in man. Basic information on the biology of the organism would show the relationship of T. gondii to other protozoa and possibly give some explanation for the parasite's unusual lack of host specificity and unique means of multiplication.

Proposed Course of Project: Emphasis will be placed on studying the sexual and asexual stages and their cultivation in vitro. Attempts to determine the factors which allow the parasite to divide by schizogony in the natural host but by endodyogeny in abnormal sites will be made. The immunological aspects of the disease in the cat will be examined to supplement existing data on the importance of cats as reservoir hosts. Studies on related organisms will be continued for comparative purposes and to examine the possibility that other coccidia would be infective for man.

Honors and Awards:

Invited lecturer on the biology of Toxoplasma gondii, NIH Graduate School.

Editor, Proceedings of the Helminthological Society of Washington

Assistant Editor, Journal of Parasitology

Member, Editorial Board, Journal of Protozoology

Publications:

Fayer, R., Melton, M. L., and Sheffield, H. G.: Quinine inhibition of host cell penetration by Toxoplasma gondii, Besnoitia jellisoni, and Sarcocystis sp. in vitro. J. Parasitol. In press.

Serial No. NIAID 125-I
1. Parasitic Diseases
2. Cell Biology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Electron Microscope Studies on Parasites

Previous Serial Number: Same

Principle Investigator: Harley G. Sheffield

Other Investigators: None

Cooperating Units: Keio University, School of Medicine, Tokyo, Japan
Imperial College Field Station, Ascot, England

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	21/12			21/12
Professional	9/12			9/12
Other	12/12			12/12

Project Description:

Objectives: To determine the fine structure of various parasites in relation to their taxonomy, life cycles, reproduction and other physiological functions.

Methods Employed: Parasites are obtained from their host animals or culture at the developmental stage desired or after appropriate experimental procedures. The parasite tissue is processed, sectioned and then studies with the electron microscope and/or cytochemical methods.

Major Findings: Tissues obtained from coccidia-free kittens which were infected with Toxoplasma gondii were studied. Additional data has been collected which demonstrates the similarities between the endogenous stages of T. gondii in the cat intestine and those of other coccidia. All stages from early trophozoite to the oocyst are intracellular and are enclosed in a parasitophorous vacuole which is surrounded by a dense membrane. This membrane

is considerably thicker than that found in other coccidian infections. The significance of this finding is, as yet, not known but the membrane undoubtably plays an important role in the successful host-parasite relationship. No evidence of deterioration has been noted in infected intestinal epithelial cells.

Studies of the microgametes of T. gondii and their formation have been completed. Microgametocytes are first recognized as large multinuclear stages with an accumulation of amylopectin granules. After the final nuclear divisions the chromatin condenses and is situated at the periphery of the microgametocyte. Centrioles can be observed between the nucleus and the plasma membrane. They later form the basal bodies of the flagella. The condensed chromatin separates from the remaining nucleoplasm and is enclosed in a bud from the microgametocyte surface. Flagella develop and a mitochondrion is enclosed. Finally, the microgamete is freed by pinching off of the plasma membrane of the microgametocyte. A residuum of microgametocyte cytoplasm remains.

The microgamete is elongate and has two flagella which originate near the anterior end and are directed posteriorly. Except for the anterior end the remainder of the microgamete is filled with the dense nuclear material and a single mitochondrion. At the anterior tip there is a dense structure with periodic banding. This structure is analagous to the perforatorium and is believed to aid in the penetration of the microgamete into the macrogamete.

Studies on the trophozoite of Lankesteria culicis have been continued in collaboration with Professor P. C. C. Garnham, Imperial College Field Station, Ascot, England. Changes in the morphology of the sporozoite after penetration have been observed. After a brief intracellular development this parasite leaves the intestinal epithelium and continues its life cycle in the lumen although it maintains an attachment to the epithelial cell by means of the mucron. The mucron is formed at the anterior end of the parasite during its intracellular development. Numerous vesicles probably originating in the Golgi apparatus migrate to the anterior region of the parasite and the material transported within them fills the mucron. The nature of the material and its function will be investigated.

A study of the cytopathological effects of infection by Trypanosoma cruzi in mouse liver cells has been initiated in collaboration with Dr. K. Asami, Department of Parasitology, School of Medicine, Keio University, Tokyo, Japan. In addition to the known lipid accumulation in the liver, preliminary results indicate that the endoplasmic reticulum is very active and its cisternae are greatly dilated.

Significance to Biomedical Research and the Program of the Institute:

Continued investigation of the similarities between Toxoplasma gondii and other coccida provides new leads to the study of the biology and epidemiology of the former. Study of the differences of development in the natural host compared to that in man and other animals could provide insight into the mechanism of host specificity in this parasite. Study of related organisms will provide additional information which could allow a better understanding of the structure of T. gondii and its relation to the functioning of the organism.

Proposed Course of the Project: Studies on the endogenous stages of T. gondii in the cat intestine will be continued. Emphasis will be placed on both the early stages of development prior to gamete formation and on the development of gametes and oocysts. Development will also be studied in vitro as techniques permit. Mechanisms of penetration of various stages will be studied and supplemented with information obtained from other parasites. Studies on Lankesteria culicis and Trypanosoma cruzi will be continued with collaborating laboratories. A portion of the time will be used to provide other investigators with electron microscopic assistance when their needs arise.

Honors and Awards:

Visiting Scientist, Keio University, Tokyo, Japan. Awarded by the Japan Society for the Promotion of Science. Jan. 15-Apr. 14, 1972.

Guest Speaker, Annual Meeting of the Japanese Society of Parasitologists, Okayama, Japan.

Publications:

Sheffield, H. G.: Fine structure of Toxoplasma gondii and related sporozoans. Jap. J. Parasitol. 21(Suppl.): 16-17, 1972.

Richards, C. S. and Sheffield, H. G.: Unique host relations and ultrastructure of a new microsporidian of the genus Coccospora infecting Biomphalaria glabrata. Proc. IVth Internat. Colloq. on Insect Path. 1970.

Serial No. NIAID 125-K
1. Parasitic Diseases
2. Parasite Growth and Differentiation
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Amebiasis studies in India

Previous Serial Number: Same

Principal Investigator: Louis S. Diamond

Other Investigators: Milford N. Lunde

Cooperating Units: Dr. Om Prakash, All-India Institute of Medical Sciences, New Delhi, India (PL 480 Project Agreement No. 01066-1)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12			2/12
Professional	2/12			2/12
Other				

Project Description:

Objectives: To evaluate an intradermal test for amebiasis using an antigen, histolyticin, from axenically cultivated Entamoeba histolytica; to determine the usefulness of the indirect hemagglutination test in assessing the effectiveness of drug therapy in amebiasis; to obtain data on the epidemiological and pathological significance of the E. histolytica-like Laredo ameba; to study the pathobiology of the clinical manifestations of chronic intestinal amebiasis and amebic hepatitis, and the relation of amebic infection and non-specific ulcerative colitis.

Methods Employed: Clinical evaluation, stool examinations, hemagglutination and agar-gel serology, skin testing, Tracerlab tracer scanner (Model Sc-47), stool, liver and pleural exudate culture.

Major Findings: A preliminary study to determine the dose range of histolyticin for detection of amebic disease was carried out on 276 individuals. Included were 103 cases of amebic disease and 173 individuals who were either in apparent good health, suffering from a variety of non-amebic parasitic disorders or with non parasitic disorders of the gut. The results indicated that the most effective dose range was between 4 and 8 μg amebic protein. In this study, based on a single skin test, no adverse reactions were observed in the dose range tested, i.e. 1 to 16 μg. However, Kirkpatrick, Lunde and Diamond (see Serial No. 33-(c)) studying cutaneous responses to histolyticin in normal volunteers who neither had a history of amebic disease nor had ever resided in endemic areas showed that a single dose of 8 μg not only produced a high number of false positives, but also sensitized the subjects so that a second test two weeks later elicited an even greater number of false reactions. On the other hand, 2 and 4 μg doses did not produce false positives.

A limited follow-up study of Indirect Hemagglutination titers (IHA) on 12 patients after treatment of their amebic liver infections with Metronidazole indicated little usefulness of the IHA test in assessing the effectiveness of chemotherapy. Significant drops in titer did not occur until many weeks after completion of successful treatment. Similar observations have been made by other investigators.

Colonic motility studies were performed on 22 individuals including 10 classified as cases of non-dysenteric amebic colitis, 2 of irritable bowel syndrome (E. histolytica negative), and 10 apparently healthy individuals. Patients with amebic colitis showed great sensitivity to external cholinergic stimuli (Prostigmine), but under basal conditions the behavior of the colon in these subjects was no different from the controls.

Significance to Bio-medical Research and the Program of the Institute:

The data presented in this and last years reports point up the usefulness of histolyticin in a skin test for amebic disease, especially the more difficult to diagnose extra-intestinal forms.

Proposed Course of the Project: The recent death of Dr. Prakash, the Principal Investigator of the PL-480 Project, has necessitated limiting the objectives of this study to its most practical aspects. An effort will be made to pin-point the optimal dose of histolyticin within the range determined by the study of Kirkpatrick et al. Emphasis will be placed on skin-testing a larger normal population, and conduct of a field trial in a village located in an endemic center.

Honors and Awards:

Dr. Diamond was a guest lecturer at the following Institutes: Statens Bacteriologiska Laboratorium, Stockholm, Sweden (Oct. 22, 1971), All-India Institute of Medical Sciences, New Delhi, India (Oct. 26, Nov. 5, 1971), Central Drug Research Institute, Lucknow, India (Nov. 9, 1971), Hadassah Medical School, Jerusalem, Israel (Nov. 18, 1971).

Publications:

Kirkpatrick, C. H., Lunde, M. N. and Diamond, L. S.: Cutaneous responses of normal subjects to histolyticin from Entamoeba histolytica. Am. J. Trop. Med. Hyg. 21: 18-21, 1972.

Serial No. NIAID 125-P
1. Parasitic Diseases
2. Cell Biology and Immunology
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunological studies on toxoplasmosis and other parasitic diseases

Previous Serial Number: Same

Principal Investigator: Milford N. Lunde

Other Investigators: Louis S. Diamond, Kendall G. Powers, and William E. Collins

Cooperating Units: Primate Malaria Unit, Chamblee, Georgia

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	19/12			19/12
Professional	14/12			14/12
Other	5/12			5/12

Project Description:

Objectives: To differentiate and characterize the immune response in acute, chronic and reactivated latent toxoplasmosis and to evaluate diagnostic methods for detecting reactivation of the disease; to evaluate the skin test and other serological procedures as aids in the diagnosis of amoebiasis; to develop and evaluate the indirect hemagglutination test for malaria; to develop a specific serological test for the detection of antibodies to nematode infections in humans.

Methods Employed: The dye, hemagglutination, (IHA), complement fixation, agar-gel diffusion, indirect fluorescent antibody (IFAT) tests are employed to determine serum antibody. Isolation of organisms is accomplished by inoculation of blood and other tissues into mice.

Sera are obtained from: a) Clinical Center, NIH, b) PL-480 project #01066-1, New Delhi, c) Perinatal Research Branch, NINDS, d) Primate Malaria Unit, e) Blood Bank, Clinical Center.

Major Findings: By means of reverse hemagglutination techniques, sensitizing erythrocytes with antibody instead of antigen, it has been possible to titrate prepared Entamoeba histolytica and Toxoplasma gondii antigens. Methods for isolation of antibody by adsorption to sensitized bentonite and subsequent elution of antibody with glycine - HCl have been developed.

Malarial antigens prepared at the Primate Malaria Unit, Chamblee, Georgia have been tested for stability under various storage conditions. The lyophilized antigen has been subjected to 100°C for 10 minutes, 56°C for 3 days and 37°C for 21 days without losing its ability to adequately sensitize erythrocytes for the indirect hemagglutination test. Repeated freezing and thawing of the antigen (more than 10X) had a harmful effect on its potency. The antigen has been partially evaluated in the IHA test for human malaria with serum from human prison volunteers, Chamblee. Thus far results indicate that the IHA test may not be as sensitive as the malaria (IFAT) especially in the early primary infection. The IHA test may be useful and more practical in epidemiological studies of large population groups.

Significance to Bio-medical Research and the Program of the Institute:

There has been much public concern in recent months about the acquisition of toxoplasmosis during pregnancy, especially when cats are present. The finding of a positive IHA titer in the serum of the pregnant female is in itself of no diagnostic help because of the high frequency (30%) of such titers in the normal adult population. The significance of toxoplasma specific IgM antibodies, especially at low levels $\leq$ 1:40 during pregnancy has not been resolved. Thus, studies were recently initiated to attempt to determine the significance of specific IgM positive titers during pregnancy.

The value of the IHA test for the detection of antibody to malaria is presently, unresolved. The antigen of choice and its methods of preparation are also unresolved. Continued work along these lines will eventually lead to a better understanding of the problems involved in sero-diagnosis and sero-epidemiology of malaria.

The combined use of pyrimethamine and sulfadiazine in the treatment of toxoplasmosis has had some hematological side effects such as leukopenia and thrombocytopenia. Pyrimethamine may also be teratogenic. These factors indicate the need for continued studies of drugs with anti-toxoplasma activity.

Proposed Course of the Project: Through the cooperation of the Primate Malaria Unit, NIAID, the sero-diagnosis of malaria, especially with the IHA test, will continue to be evaluated with the aim of clarifying some of the currently unresolved questions.

Studies recently initiated to determine the significance of toxoplasma-specific IgM antibody in congenital toxoplasmosis will be continued.

Chemotherapeutic studies on the effectiveness of chlorinated lincomycin analogues against Toxoplasma will be pursued to determine the effectiveness of these drugs against the cyst stage of the parasite.

The development of a specific serological test for the detection of antibodies to Angiostrongylus cantonensis, the causative agent of eosinophilic meningitis in the Pacific, will be initiated.

Additional sera from PL-480 project 01066-1 in India will be examined for anti E. histolytica antibodies.

Honors and Awards: None

Publications:

Lunde, M. N. and Gelderman, A. H.: Resistance of AKR mice to lymphoid leukemia associated with a chronic protozoan infection. J. Natl. Cancer Inst. 47: 485-488, 1971.

Kirkpatrick, C. H., Lunde, M. N., and Diamond, L. S.: Cutaneous responses of normal subjects to histolyticin from Entamoeba histolytica. Am. J. Trop. Med. Hyg. 21: 18-21, 1972.

Serial No. NIAID 132

1. Parasitic Diseases
2. Malaria
   a. Primate Malaria
   b. Human Malaria
3. Bethesda, Maryland and Chamblee-Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30 1972

Project Title: Administration and Planning of Section

Previous Serial Number: NIAID 133-I

Principal Investigators: Peter G. Contacos and Louis H. Miller

Other Investigators: William E. Collins and William A. Howard

Cooperating Units: None

Man Years:

	NIAID Lab. S aff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	30/12			30/12
Professional	6/12			6/12
Other	24/12			24/12

Project Description:

On February 28, 1970 the Section on Chemotherapy and the Section on Primate Malaria were combined and named the Section on Malaria which has two field units, the Unit on Primate Malaria under Dr. William E. Collins and the Unit on Human Malaria at the Atlanta Federal Prison under Dr. William A. Howard.

Support and organization for the section is directed through the Section and unit heads. The professionals on this project are responsible for the overall planning and coordination of research in the Section. Dr. Miller closely supervises the patient care at the Human Volunteer Unit at the Atlanta prison. Supervision over personnel and the maintenance of the unit is handled by the professionals on this project.

During the past year Dr. Peter G. Contacos transferred from the NIH to the Center for Disease Control, Atlanta. Dr. W. A. Krotoski transferred from the human malaria unit to the primate malaria unit. Dr. William A. Howard became medical officer in charge of the human malaria unit and Dr. Richard Glew joined the unit as medical officer. Miss Elizabeth Guinn retired from the NIH.

Consultative arrangements continue at the human malaria unit through Emory University, School of Medicine, although the arrangement with Dr. Edmund Farrar of the Department of Preventive Medicine has been discontinued because he is no longer at Emory University. Dr. Galambos made frequent consultative visits to the unit.

Cooperative studies continue with Scientists at the Central American Malaria Research Station of the Center for Disease Control.

Honors and Awards: See under individual scientific projects

Publications. See under individual scientific projects.

Serial No. NIAID 132-A
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic aspects of some protozoal infections in laboratory animals

Previous Serial Number: NIAID 125-Q

Principal Investigator: Robert Herman

Other Investigators: Tsugiye Shiroishi

Cooperating Units: Laboratory of Viral Diseases (NIAID)
Viral Leukemia and Lymphoma Branch (NCI)

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	24/12			24/12
Professional	12/12			12/12
Other	12/12			12/12

Project Description:

Objectives: To evaluate the role of virus-induced interferon as the possible protective mechanism in viral inhibition of exo-erythrocytic forms of the avian malarial parasite, Plasmodium gallinaceum in chick embryos (Herman, Shiroishi, Buchler and Baron).

To determine the specificity of the immunosuppressive effects of various agents on the different parasitic forms of avian malaria in sporozoite-initiated malaria (P. gallinaceum) (Herman and Shiroishi).

To evaluate the effects of a self-limiting strain of rodent malaria, P. berghei, upon virus-induced tumors and leukemia in mice (Buswell, Gazdar and Herman).

Methods Employed: 1) The methods employed to evaluate the effects of two viruses (i.e., Chikungunya and Newcastle disease virus (NDV) upon the exo-erythrocytic forms of avian malaria (P. gallinaceum) are essentially the same as in the previous Annual Report (1970-1971). Additional work has been completed in attempting to protect chicks against P. gallinaceum by injections of NDV. Further work has been carried on using whole embryo extracts as well as allantoic fluid for interferon assays.

2) By timed injections of the immunosuppressant, cyclophosphamide into chicks and by x-irradiation of chicks, followed by infection with sporozoites of P. gallinaceum, the specific immunosuppressive effects of the treatments upon the different exo-erythrocytic stages of the parasite are being evaluated. Evaluation is by stained smears of parts of infected organs and by subinoculation of blood from infected chickens to normal chickens at critical times during the pre-erythrocytic stages of the infection.

3) Mice injected with the self-limiting strain of P. berghei (17X) and then injected with either mouse sarcoma virus (MSV) or mouse leukemia virus (MLV) have been followed with regard to parasitemias by stained blood smears. In addition, the effects of malaria upon tumor formation and leukemia have been evaluated.

Major Findings: 1) The effect of viral injection on subsequent infection by the avian malaria parasite, P. gallinaceum, was that, in ovo, Chikungunya virus injection protected embryos against P. gallinaceum multiplication. This protection was associated with induction of strong interferon-like resistance but minimal amounts of interferon. In comparison, NDV provided no protection and induced neither interferon-like resistance nor interferon in ovo. In young chicks NDV is protective and is known to induce interferon in the hatched chick. Although sufficient quantities of chicken interferon are not available to directly test the role of interferon, the present findings are consistent with the previously reported protection by interferon against murine malaria.

2) When cyclophosphamide is injected into young chicks which are then infected with sporozoites of P. gallinaceum two days later, the course of infection is much enchanced over controls. This enhancement is apparently due to an increased reproduction of one of the pre-erythrocytic stages of the parasite. Cyclophosphamide, like x-irradiation, suppresses the induction of acquired immunity of chickens to blood-induced P. gallinaceum infection. Young chicks injected with cyclophosphamide and infected with sporozoites of P. gallinaceum have a greater number of exo-erythrocytic stages of the parasite in their tissues and in their blood at certain times after infection. This immunosuppressant apparently has no effect upon the "tissue-blood barrier" in P. gallinaceum infections nor upon the penetration of merozoites into red blood cells. The suppressive effect appears to function at the cellular level, i.e., with the endothelial cells of capillaries which are host to certain exo-erythrocytic stages of the parasite.

3) Mice injected with either mouse sarcoma virus (MSV) or mouse leukemia virus (MLV) followed by infection with the 17X strain of P. berghei showed prolonged and enhanced parasitemia when compared to mice not injected with the virus. Some mice even died of malaria. Apparently the malarial infection has little, if any, untoward effect upon either the tumors or leukemia produced by these viruses, respectively.

Significance to Bio-medical Research and the Program of the Institute:

The use of chick embryos to evaluate the possible effects of interferon upon the exo-erythrocytic stages of malaria is of considerable importance. Although primate and rodent malarias differ in certain respects from avian malarias, they retain certain basic similarities regarding their courses of infection.

The study of exo-erythrocytic stages of avian malaria offers certain advantages over that of other malarial species in that they are easily cultured in vitro and easily detectable within various host tissues. The chick embryo, because of its relative immunological incompetence, offers further advantages for the study of these forms. The fact that interferon may possibly inhibit the reproduction of exo-erythrocytic stages may prove what has only been speculated in rodent malaria studies.

Similarly, the effects of cyclophosphamide upon the exo-erythrocytic stages of P. gallinaceum may have application to human malaria. Again, because of the ease of evaluating the effects of this agent upon exo-erythrocytic forms, the avian parasite is most adaptable to use for quantitative studies.

Although P. berghei (17X strain) has no apparent effect on the course of MSV or MLV in the mouse, the fact that these viruses do prolong and enhance the parasitemias in both cases is of significance. To what extent individuals with immunological defects respond to malarial infection is not specifically defined. Through these studies using a variety of immuno-suppressants (including certain viruses) the effect of such immunosuppression in man upon malaria may be considered.

Proposed Course of the Project: None. Expiration of Senior Staff Fellowship.

Honors and Awards:

Invited seminar speaker. Department of Microbiology, State University of New York at Buffalo. November 4, 1971.

Invited seminar speaker. Department of Zoology, Rutgers, The State University, New Brunswick, New Jersey. January 20, 1972.

Invited seminar speaker. Department of Microbiology, Medical College of Virginia, Richmond, Virginia. February 8, 1972.

Publications:

Schmunis, G. A. and Herman, R.: Macrophage activity of thymectomized mice infected with Leishmania donovani. Experientia, 27: 602-604, 1971.

Herman, R. and Baron, S.: Immunologic-mediated protection of Trypanosoma congolense-infected mice by polyribonucleotides. J. Protozool. 18: 661-666, 1971.

Herman, R.: The effects of interferon and its inducers on experimental protozoan parasitic infections. Trans. N. Y. Acad. Sci. II 34: 176-180, 1972.

Serial No. NIAID 132-B
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Immunity and Pathology of Malaria

Previous Serial Number: NIAID 131-C

Principal Investigators: Kendall G. Powers and Louis H. Miller

Other Investigators: John F. Finerty

Cooperating Units: Laboratory of Microbial Immunity, NIAID

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	18/12	3/12		21/12
Professional	10/12	2/12		12/12
Other	8/12	1/12		9/12

Project Description:

Objectives: To study the immune response and pathological consequences of acute and chronic malaria and other parasitic diseases.

Methods Employed: Various host-parasite models are used in these studies including Plasmodium knowlesi and P. cynomolgi in rhesus monkeys, P. falciparum in owl monkeys and P. berghei and P. vinckei in rodents. Immunologic studies involve qualitative and quantitative changes in immunoglobulins as well as changes in antibody titer measured by the schizont agglutination test and the fluorescent antibody test. Mechanisms of cellular immunity are being studied with special emphasis on lymphocyte function and the role played by the erythrocyte membrane in this type of immunity. Rosettes, formed by parasites adhering to lymphocytes, are being investigated to determine the mechanism involved in this reaction. Particular emphasis is placed on determining the stage of infection when rosettes are formed, if antibodies or immunoglobulins are involved, the specificity of the reaction, i.e., is it strain or species specific, and the possibility of using a test such as this to detect the

immunologic status of the host. Chemistry of the membrane of malaria infected erythrocytes are investigated using electron dense colloids.

Major Findings:

1. Schizont agglutination test. The schizont agglutination technique is being investigated as a method for studying the host immune response in malaria. Several modifications of the originally described technique have been made such as the separation of the schizont-infected erythrocytes from the blood using Ficoll gradients, the use of micro amounts (0.025 ml) of antigen and test sera instead of 0.4 ml of each component and the preservation of antigenic material using various fixatives. The sensitivity of the modified test does not seem to be deminished and in some instances it appears to be enhanced when compared to the original method. When compared with the fluorescent antibody test it detects malarial antibody at about the same time in the early infection and whereas fluorescent antibody reciprocal titers in P. knowlesi-infected monkeys reach about 1:256, the modified schizont agglutination test, on the same sera, gives titers in excess of 1:500,000. Other investigators have reported that the original test can detect antigenic variation within a malarial strain. We currently are investigating this concept. We have preliminary data which indicates that fixation and lysis of the schizont-infected erythrocyte produces an antigen which does not change with time and one that is more reactive than unfixed material.

2. Rosette formation. During the course of studies on P. falciparum in owl monkeys we observed that malarial parasites arranged around lymphocytes in a rosette fashion. This only was observed when blood was packed by centrifugation and allowed to remain in this state for 15-30 minutes, either at room temperature or at 37° C. Subsequently we were able to produce this phenomen in blood infected with P. berghei and P. knowlesi. (For details see Dr. Finerty's report, 117-C).

3. Chemical organization of the membrane of malaria infected red cells. Studies, initiated at Columbia University, were completed at the NIH on the organization of the lipid micelle of malaria infected red cells. Positively charged ferric oxide colloid, suspended in propanoic or butanoic acid bound to the membrane of infected red cells in a pattern different from uninfected red cells. The frequency and size of colloid aggregates were greatest over schizont infected red cells. The effects of osmium fixation, lipid extraction, saponification and phospholipase A on the differential binding on infected red cells are consistent with the notion that the lipid portion of the membrane, and in particular the phospholipids, are different in infected red cells. It is proposed that the acyl groups of propanoic acid intercolate into the hydrophobic region of the membrane between the acyl group of adjacent phospholipids and that the carboxyl group of propanoic acid is within the hydrophilic region. The difference in colloid binding between infected and uninfected red cells raises the question of how the structure of the membrane regulates interaction between the cell and circulating substances.

Findings on the effects of various strains of Schistosoma mansoni in rhesus monkeys are included in report No. 121-H.

Significance to Bio-medical Research and the Program of the Institute:

During malarial infection, the host produces antibodies to infected red cells that are coated with malarial antigens. Probably more important for functional immunity are the antigens present on the surface of merozoites. Circulating antibody, with or without complement, could effectively destroy the parasite or block its infectivity to red cells.

If there are many antigenic variants, as claimed by Brown and others, then the problem of immunization becomes more difficult, even if immunogens can be identified. It is hoped that these studies will develop methods to classify the antigenic variants, to separate the antigens on the surface of red cells and to identify parasites responsible for immunity.

We have preliminary data which indicate that fixation of schizont-infected red cells produces a stabile, sensitive antigen. It is possible that this antigen may be used in the rapid detection of malarial antibodies in endemic areas.

Proposed course of the project: Work on the schizont-infected erythrocyte agglutination test will be continued with emphasis on reproducability and on the sensitivity of the test to detect antigenic differences in parasite populations. We also plan to evaluate the usefulness of this test system in animals infected with either Plasmodium falciparum, P. cynomolgi or P. berghei.

To date there is no method for obtaining a preparation of parasites free from red cell membranes. Red cells infected by malarial parasites at different stages of asexual development were lysed by various methods. The completeness of separation will be monitored by electron microscopy and interference light microscopy.

Honors and Awards:

Powers, K. G. and Cheever, A. W.: Comparison of geographic strains of Schistosoma mansoni in the rhesus monkey. Bull. Wld. Hlth. Org. (In Press).

Cheever, A. W. and Powers, K. G.: Schistosoma mansoni infection in rhesus monkeys: comparison of the course of heavy and light infections. Bull. Wld. Hlth. Org. (In Press).

Serial No. NIAID 132-C
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Experimental Chemotherapy of Malaria

Previous Serial Number: NIAID-131

Principal Investigator: Kendall G. Powers

Other Investigators: Richard L. Jacobs

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	14/12			14/12
Professional	7/12			7/12
Other	7/12			7/12

Project Description:

Objectives: To establish and maintain a series of models for use in evaluating the potential antimalarial activity of various compounds. This would include parasites from birds, rodents and non-human primates. Drug activity is evaluated against both blood and tissue stages of the parasite.

Methods Employed: Compounds with antimalarial activity are first tested orally and intraperitoneally in mice infected with Plasmodium berghei and in certain instances in mice infected with P. vinckei. Efficacy is based on the percentage of infected erythrocytes in treated mice as compared to untreated controls. Compounds may also be evaluated against strains of P. berghei resistant to chloroquine, pyrimethamine, quinine, DDS or certain long-acting sulfonamides and against P. vinckei resistant to pyrimethamine and chloroquine. When activity warrants, compounds are evaluated against blood- and sporozoite-inoculated P. gallinaceum infections in chickens and against P. cynomolgi, P. knowlesi and/or P. coatneyi infections in rhesus monkeys.

A new host parasite system is currently being used to further evaluate compounds that have demonstrated antimalarial activity. Aotus trivirgatus (owl monkey) infected with a chloroquine-resistant strain of P. falciparum is now available for use in the search for compounds that are efficacious against chloroquine-resistant falciparum malaria in man.

Major Findings: Two chlorinated lincomycin analogues were tested and found active against blood induced chloroquine-resistant falciparum malaria in owl monkeys. Acute infections were cured with 7 daily oral doses of either 75 mg/kg of clindamycin hydrochloride (U-21) or 10 mg/kg of N-demethyl-4'-pentyl clindamycin hydrochloride (U-24). No animal was considered cured until it had remained negative for the parasite for at least 30 days after splenectomy. Although both compounds were relatively slow in clearing fulminating infections from the blood (7 days), none of the treated monkeys died as a result of infection nor were any signs of drug toxicity apparent during a 100 day observation period. U-21 is commercially available and relatively nontoxic whereas tests for human toxicity of U-24 are still incomplete and the drug is not yet approved for human use.

The radical curative activity of one of the lincomycin analogues was evaluated in rhesus monkeys infected with P. cynomolgi sporozoites. Shortly after patency, groups consisting of 6 monkeys each were treated with 7 daily oral doses of either 50 or 100 mg/kg of U-24. Although parasites disappeared from the blood by the end of the treatment period, relapses eventually occurred in all monkeys, even after chloroquine therapy, indicating that these regimens were ineffective against late exoerythrocytic stages of the parasite. A similar study was conducted to determine the radical curative activity of this compound in monkeys with sporozoite-induced chronic infections of 8 months duration. The drug was also ineffective in this experiment.

The antimalarial activity of U-24 against different stages of P. gallinaceum was also investigated. This compound possesses definite sporozoicidal activity in this model. Sporozoites were inactivated when incubated in sera from treated birds for either 15 or 30 minutes at 37°C. Sporozoites incubated for the same period in untreated sera were uniformly infective. A single 50 mg/kg oral dose of U-24, given 1-6 days before sporozoite injection, protected 100% of the chickens from infection. This same single dose cured all birds when pre-erythrocytic tissue parasites were treated either 12, 24, 36 or 48 hours after sporozoite injection. The drug was relatively ineffective against erythrocytic stages of the parasite but treatment during the acute stage of infection prevented the development of exoerythrocytic parasites in the brain.

Significance to Bio-medical Research and the Program of the Institute:

There is a world-wide interest in the discovery of effective compounds for the treatment of chloroquine-resistant falciparum malaria in man. Particular emphasis has been placed on identifying drugs which differ chemically and in mode of action from commonly used antimalarials. The clorinated lincomycin analogues are most promising compounds in this regard. Their curative activity

against a strain of falciparum malaria in owl monkeys, which is highly resistant to chloroquine, suggests that they will also be effective against this parasite in man. Clindamycin (U-21) is of particular interest since it is relatively nontoxic and is already commercially available for use in humans as an antibacterial agent.

Proposed Course of the Project: Investigations have been completed on the antimalarial activity of the lincomycin analogues in experimental animals. Because of the information from these studies, trials of clindamycin against falciparum malaria in man are now underway (NIAID 134-A). The capability for evaluating promising compounds for antimalarial activity will be maintained.

Honors and Awards:

Secretary-Treasurer: Tropical Medicine Association of Washington, D.C.

Chairman: NIAID Safety Committee, NIH, Bethesda, Maryland.

Executive Committee member: Helminthological Society of Washington.

Publications:

Powers, K. G. and Jacobs, R. L.: Activity of two chlorinated lincomycin analogues against chloroquine-resistant falciparum malaria in owl monkeys. Antimicrob. Ag. Chem. 1: 49-53, 1972.

Serial No. NIAID 132-D
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Drug Resistance in Experimental Malaria

Previous Serial Number: NIAID 131-B

Principal Investigator: Richard L. Jacobs

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	10/12			10/12
Professional	5/12			5/12
Other	5/12			5/12

Project Description:

Objectives: To investigate the mechanisms of acquired drug resistance in rodent malarial parasites; to characterize different drug-resistant strains by their cross-resistant patterns to various antimalarial drugs, morphology, pathogenicity, and stability of resistance in the absence of selective drug pressure.

Methods Employed: Various strains of Plasmodium berghei are maintained by blood transfer in mice. Gametocyte producing strains are maintained by passage through mice, hamsters, and Anopheles stephensi. Prior to the selection of resistant strains, standard dose-suppression tests were conducted to establish sensitivity of the parent strain of P. berghei to the various drugs under study. Parasitemia was followed in blood smears stained by the Giemsa procedure. The suppressive effect of different doses of drug was determined by comparison of mean percent parasitemia in treated groups with the untreated control group of mice.

The procedure used to develop drug-resistant strains of P. berghei involved the repeated blood passages of the parasite in mice treated with the highest dose of drug that allowed for some development of parasitemia during each passage. Transfer to fresh mice was made as rapidly as possible, usually before day 7, using large numbers of parasitized erythrocytes (up to 20 million per mouse) which were inoculated intraveneously. Drug dosages were increased as indicated by parasite development in the previous passage in order to maintain the maximum degree of selective drug pressure. Parasite strains were considered totally resistant when they devloped in mice treated with the maximum tolerated dose (MTD) of the drug. Resistant strains were subsequently maintained in mice treated with the MTD of drug during each subpassage. In order to assess the stability of resistance in the absence of selective drug passage, the resistant strains were repeatedly subpassaged in untreated mice and tested periodically for drug sensitivity, changes in morphology, virulence, and other characteristics associated with resistance.

Major Findings: Using the above procedure which previously resulted in the development of resistant strains of P. berghei to commonly used antimalarials (chloroquine, quinine, pyrimethamine and primaquine), strains are currently being developed that are resistant to clindamycin hydrochloride and to minocycline.

During 14 successive passages in mice treated with U-21, a four-fold increase in resistance has been observed. The parent strain was almost suppressed by four daily doses of 25 mg/kg, but after 14 treated passages over a period of 73 days the strain develops in mice treated with daily doses of 100 mg/kg. This strain is being maintained in treated mice to obtain a higher degree of resistance to clindamycin. After 13 successive passages in mice treated with clindamycin, this strain was found to be normally sensitive to minocycline. Resistance of P. berghei to clindamycin developed slowly, whereas resistance to conventional antimalarials developed more rapidly. Strains totally resistant to chloroquine, quinine, pyrimethamine and primaquine were developed in eight to twelve passages. No morphological changes or alteration of virulence (in untreated mice) have been observed in the strain as resistance to clindamycin has developed.

The data on resistance to minocycline are more preliminary, since only four passages in treated mice have been completed. The parent strain tolerated daily doses of 100 mg/kg, but daily doses were increased to 200 mg/kg during passages two through four. This suggests that resistance to minocycline may develop more rapidly than to clindamycin.

Significance to Biomedical Research and the Program of the Institute:

Drug-resistant strains of malaria, particularly resistance to chloroquine, continue to present a world-wide problem in the chemotherapy of malaria. Strains of P. falciparum that are resistant to all of the commonly used synthetic antimalarials, as well as to quinine, continue to be encountered in widely separated areas of the world. Also, other species of malaria that infect man may become refractory to presently effective drugs. The appearance

of drug-resistance in malarial parasites of man has served to emphasize t he need for a better understanding of the basic mechanisms of drug action on the parasite and other aspects of host-drug-parasite relationships. Plasmodium berghei has proven to be a valuable laboratory model for the investigation of the mode of action of various antimalarial compounds and some of the basic aspects of acquired drug resistance in malarial parasites.

Recent reports regarding the successful use of antibiotics, especially lincomycin and tetracycline derivatives, for the treatment of drug-resistant malaria in man raise questions regarding the prolonged usefulness of these compounds for this purpose. If no cross resistance occurs, then resistance to one will not invalidate the usefulness of the other compound. Studies in mice infected with P. berghei should give some insight regarding the ease of development of resistance to these compounds, cross-resistance patterns and stability of resistance once it is developed. These results should be useful in predicting the future usefulness of the antibiotic derivatives for the treatment of malaria in man.

Proposed Course of the Project: These studies will be continued with the objective being the development of strains of P. berghei totally resistant to these lincomycin and tetracycline derivatives. Cross-resistance patterns can then be compared to various antimalarial drugs as well as the two antibiotics. Stability of resistance in the absence of drug will be investigated. Other features of the antibiotic-resistant strains, such as morphological changes and alterations in virulence will also be studied.

Serial No. NIAID 132-E
1. Parasitic Diseases
2. Malaria
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on the Labelling of Sporozoites of Malaria

Previous Serial Number: None

Principal Investigators: Richard L. Jacobs and Louis H. Miller

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	11/12			11/12
Professional	6/12			6/12
Other	5/12			5/12

Project Description:

Objectives: To develop methods for the labelling of sporozoites in order to determine their distribution in the mammalian host (kinetics) and the development from the sporozoite through the exoerythrocytic stages within the hepatic parenchymal cells. The effect of drugs and immunity on the kinetics of sporozoite distribution and the developing EE forms will be studied.

Methods Employed: The NK-65 strain of Plasmodium berghei is maintained in cyclical passage through mosquitoes (Anopheles stephensi), mice and hamsters. Sporozoites are harvested by dissection of infected mosquito salivary glands at appropriate times after the infective blood meal. Sporozoites are exposed in vitro to fluorescent dyes and then tested for adequate labelling under the UV scope and viability by intravenous injection into susceptible hosts. In addition sporozoites will be labelled in vivo by injection of mosquitoes with a radioactive isotope at appropriate times after an infective blood meal.

The radioactive sporozoite will be harvested and injected into a susceptible host. Its development will be followed in tissue sections by autoradiography and its distribution in the host by scintilation counting of tissue homogenates from various organs.

Major Findings: Various fluorescent labels, including fluorescein isothiocyanate, rhodamine B isothiocyanate, berberine HCl and acriflavin have been used to label sporozoites in vivo. Of these compounds, fluoroscein isothiocyanate (FITC) has given superior fluorescent labelling and work has been concentrated on this compound. Batches of sporozoites labelled with FITC have been shown to be infective, but the uniformity of labelling is not known at present. Therefore, the possibility exists that the viable sporozoites in the mixture may not have taken up the fluorescent stain. The influence of temperature, exposure time, pH and buffer solution on fluorescein labelling are now being studied.

Significance to Biomedical Research and the Program of the Institute:

Probably the greatest gap in our knowledge of the life cycle of malaria lies in the mechanism by which relapses occur. The generally accepted explanation involving the reinvasion of hepatic cells by merozoites liberated from the exoerythrocytic cycle is not proven. More recently, it has been suggested that some sporozoites remain dormant for long periods of time within the hepatic parenchymal cells before developing into exoerythrocytic forms. It is hoped that the present study will determine whether late EE forms in P. vivax and P. ovole are due to slow development from sporozoites or from invasion of merozoites from the primary EE form to the secondary EE forms. This study should have a great impact on chemotherapy of hepatic schizonts (causal prophylaxis and radical cure).

Proposed Course of the Project: These studies will be continued with major emphasis on radioactive labelling of sporozoites in vivo and following the development of labelled sporozoites in the mammalian host.

Serial No. NIAID 132-O
1. Parasitic Diseases
2. Chemotherapy (later Cell Biology & Immunology)
3. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of disease (Chagas' disease) resulting from infection with Trypanosoma cruzi

Previous Serial Number: 131-O

Principal Investigator: Franklin A. Neva

Other Investigators: Dr. Gustavo Parajon of Hospital Baustista in Managua, Nicaragua

Cooperating Units: Middle America Research Unit, Canal Zone, Panama; Ministry of Health of Nicaragua.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	21/12			21/12
Professional	2/12			2/12
Other	19/12			19/12

Project Description:

Objectives: To elucidate some of the factors that determine why infection with T. cruzi is associated in some individuals with either acute or chronic disease. To develop improved serologic procedures for diagnosis of infection and/or disease due to T. cruzi.

Methods Employed: A number of strains of trypanosomes are maintained in culture, others by syringe passage, and some are maintained in serial passage alternating between mice and vector bugs. The biologic characteristics of strains kept in mouse-bug passage are also tested at intervals by checking peripheral parasitemia and histopathology of mice. Colonies of two species of triatomid bugs are maintained. One strain of T. cruzi is passaged in cell cultures of embryonic beef tissue, and tissue phases

of the parasite (mainly amastigotes and trypomastigotes) are harvested for antigen. Complement-fixation and hemagglutination tests are done in a standard manner. On follow-up visits the principal investigator and/or Dr. Parajon have gone to the study areas to locate patients who have been selected on the basis of the screening examination as having definite or suspected evidence of heart disease. In several follow-up examinations additional blood specimens and, in some instances, xeno-diagnosis tests have been performed, and electrocardiograms have been obtained.

Major Findings:

Field Study in Nicaragua: The Nicaraguan survey serum samples were all tested with one conventional crithidial-type antigen in addition to Fife's purified protein crithidial antigen, and a proportion of the serum samples were also tested against a conventional crithidial antigen prepared from a different strain of T. cruzi. The CF results obtained with our tissue culture antigen gave similar results to those obtained with Fife's purified protein antigen but serum titers tended to be higher with the tissue culture antigen. A small number of serum samples gave positive reactions to the tissue culture antigen but negative reactions with the purified protein antigen. The conventional crithidial antigens gave a high proportion of non-specific positive results. Several follow-up visits to Nicaragua have been made by the senior investigator, the last one in February 1972, and a separate follow-up visit by Dr. Parajon in June 1971. Practically all of the adults who were selected on the initial screening examination as having known or suspected heart disease have been seen on at least one follow-up examination for clinical re-evaluation, and many of them have had additional blood samples, blood cultures, or xeno-diagnosis tests performed. A final clinical evaluation for presence or absence of heart disease and for the clinical variety of heart disease is being prepared by Dr. Parajon under contract support. These results will be correlated with the serologic test results in order to assess the prevalence of heart disease that can be attributed to infection with T. cruzi and to determine some crude prevalence rates for heart disease in the study areas.

Antigen from T.cruzi from infected cell cultures: The presumably false positive reactions obtained with occasional batches of our tissue culture antigen when tested with serum from patients with cutaneous leishmaniasis still remains a problem. When positive tests are obtained with sera from individuals indigenous to Central or South America a concomittant reactivity of a specific nature to T. cruzi can not easily be ruled out. Cross-reactivity can be checked with sera from patients with Oriental sore where exposure to T. cruzi does not exist, but it appears that the cross-reactivity is more commonly seen in sera from patients with New World-type leishmaniaisis. Serum samples obtained from the Army group at the Middle America Research Unit from American military personnel with limited exposure in that region and laboratory verified mucocutaneous leishmaniasis were obtained and will be used to assess cross-reactivity

of our tissue culture T. cruzi antigen. Some evidence indicates that our tissue culture antigens which do give false positive cross-reactions are contaminated with excessive numbers of beef embryo culture cells and that this causes the cross-reactivity.

Comparative behavior of different strains of T. cruzi: The data from previous year's work has not yet been put together for publication. The analysis of histopathologic findings in infected mice has not yet been made with the different strains of T. cruzi that were studied. Several strains of T. cruzi continue to be passaged alternately in mice and in triatomid bugs and these strains are periodically checked for their infectivity for mice to add longer term observations to the results. Also, some strains of T. cruzi isolated from sylvatic sites in Panama are being maintained in a similar manner.

Significance to Bio-medical Research and the Program of the Institute:

The tissue culture antigen for serodiagnosis of T. cruzi infection appears to be equal to or greater in sensitivity to the best available antigen prepared from crithidia and hitherto considered a standard antigen. More study of the specificity of the tissue culture antigen is still needed. There is reason to believe that the tissue culture antigen might have some advantages to the conventional antigens in selected instances. Our field experience in Nicaragua has provided a model for evaluation of the importance of T. cruzi infection as a cause of heart disease in communities. Even though our study in Nicaragua had deficiencies as far as precision is concerned, it has served to provide a basis that may be recommended in the future for other groups and countries to assess the significance of this parasite as a public health problem.

Proposed Course of the Project: The field survey in Nicaragua will be completed and prepared for publication. It is hoped that the data on biologic differences in strains of T. cruzi can also be assembled for publication. It is planned to continue to assess the specificity of the tissue culture antigen as a immunologic test for infection with T. cruzi and to get more information on the reproducibility of different lots of antigen and their stability. Some of the studies on differences in strains of T. cruzi will be continued.

Serial No. NIAID 133
1. Parasitic Diseases
2. Malaria - Primate and Human Malaria
3. Chamblee & Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Susceptibility of Anopheline Mosquitoes to Simian and Human Malaria Infections and Their Ability to Transmit to Simian and Human Hosts.

Previous Serial Number: Same

Principal Investigators: William E. Collins and Peter G. Contacos

Other Investigators: Wojciech A. Krotoski, William A. Howard and Richard H. Glew

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	61/12			61/12
Professional	5/12			5/12
Other	56/12			56/12

Project Description:

Objectives: (1) To determine the relative susceptibility of anopheline mosquitoes to simian and human malaria infection; (2) to determine the rate and degree of parasite development within various mosquito hosts; (3) to attempt transmission of the various simian malarias to a wide spectrum of simian hosts by the bites of infected mosquitoes; and (4) to provide mosquitoes infected with human malaria for transmission studies in prison inmate volunteers.

Methods Employed: Isolates of simian malaria are maintained either in monkey hosts or in the frozen state for use in transmission studies. Malaria species under study include Plasmodium brasilianum (2 strains), P. cynomolgi (9 strains), P. coatneyi, P. fieldi (3 strains), P. simiovale, P. simium, P. fragile (2 strains), P. gonderi, P. knowlesi (4 strains),

P. inui (17 strains), P. jefferyi and P. hylobati. Studies are also made on P. falciparum (6 strains), P. vivax (10 strains) and P. malariae maintained in monkeys. Various species of Anopheles (A. maculatus, A. stephensi, A. freeborni, A. atroparvus, A. albimanus, A. quadrimaculatus and A. balabacensis balabacensis) mosquitoes are allowed to feed on infected simian and human hosts. Infection potential to a given mosquito species of a given malaria strain in a variety of hosts is determined and transmission by direct mosquito bites are made. Occasionally, when numbers of mosquitoes are low and gland dissections are positive, attempts to infect monkeys are made by intravenous inoculation of sporozoites from gland dissections. Mosquito susceptibility studies with the human malarias are also conducted in cooperation with the Unit on Human Malaria at the U. S. Penitentiary, Atlanta, Georgia.

Major Findings: The results of the transmission studies with various malarias are as follows:

P. knowlesi - H strain: Transmission was obtained on 41 occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 6 to 9 days with a mean of 7.5 days.

P. cynomolgi bastianellii: Transmission was obtained on 21 occasions with A. maculatus and A. B. balabacensis mosquitoes. The prepatent periods ranged from 8 th 27 days with a mean of 10.3 days.

P. cynomolgi - RO strain: Transmission was obtained on 3 occasions with A. freeborni mosquitoes. The prepatent periods were 9, 9, and 10 days.

P. cynomolgi ceylonensis: Transmission was obtained on one occasion with a triturated suspension of infected A. stephensi mosquitoes which had been stored frozen at -78° C for 1765 days.

P. coatneyi: Transmission was obtained on 9 occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 10 to 13 days with a mean of 11.7 days.

P. inui - Mulligan strain: Transmission was obtained on 6 occasions with A. b. balabacensis mosquitoes. The prepatent periods ranged from 15 to 16 days with a mean of 15.7 days.

P. vivax - Salvador II strain: Transmission from one monkey to another was obtained with A. maculatus once and with A. freeborni 5 times. The prepatent periods ranged from 14 to 44 days with a mean of 29.7 days.

P. vivax - Chesson strain: Transmission from a human volunteer to a monkey was obtained with A. maculatus mosquitoes. The prepatent period was 32 days.

P. falciparum - Cambodian I strain: Transmission to a monkey was obtained once with the bites of A. freeborni mosquitoes. The prepatent period was 36 days.

Emphasis was made on the linear sporozoite passage of the Salvador II strain of P. vivax in Aotus monkeys. After 7 passages, however, the prepatent periods continued to be considerably extended over those in man and the successful transmission rate continued below 50 percent.

A series of monkeys inoculated with very low numbers of P. cynomolgi sporozoites indicated that 10 or more were necessary to initiate infection. There was a direct relationship between the frequency of relapses and the number of sporozoites introduced. The occurrence of "late" relapses in animals with inoculations of 20 sporozoites or less suggested that certain sporozoites are programed to initiate infection at a late date thus supporting the opinion that relapses are not caused by cyclic reinvasion of the liver tissue by merozoites from maturing EE bodies.

Significance to Bio-medical Research and the Program of the Institute:

This project is part of an integrated program of the Laboratory of Parasitic Diseases in the study of simian malaria in monkeys and its relationship to man and human malaria. A clear-cut delineation of the parasite-vector relationship is essential to the proper evaluation of the potential significance of simian malarias as zoonoses in human populations. Detailed laboratory studies on the biology of the simian parasites in the mosquito host are necessary as a supplement to the work being done by other units. Further, there is need for varied species of simian malaria, simulating all of the human species in life pattern and morphology, to carry on significant studies on the chemotherapy, immunology and other aspects of malaria research. A full exposition of the host-parasite-vector relationships of the available monkey parasites is a necessity in providing these useful host-parasite combi nations. The availability of infections of human malarias in monkeys makes it possible to study the effect of drugs on the ability of these parasites to infect mosquitoes. It serves also as a source of P. vivax, P. malariae, and P. falciparum infected mosquitoes for use in drug studies in man.

Proposed Course of the Project: These studies will be conducted and expanded to include the evaluation of a number of anopheline species of mosquitoes in relation to their potentials as vectors of species of simian and human malaria. Efforts will be directed towards obtaining large numbers of gland positive mosquitoes of several simian species of malaria for use in inoculating monkeys for tissue parasite studies. Efforts will be continued towards developing more effective and efficient means not only of producing larger numbers of mosquitoes for laboratory transmission studies but also of increasing the longevity of such mosquitoes.

Publications:

Collins, W. E., Contacos, P. G., Skinner, J. C., and Guinn, E. G.: Studies on the transmission of simian malaria. IV. Further studies on the transmission of Plasmodium knowlesi by Anopheles balabacensis balabacensis. J. Parasit., 57: 961-966, 1971.

Collins, W. E., Contacos, P. G., Garnham, P. C. C., Warren, McW., and Skinner, J. C.: Plasmodium hylobati: A malaria parasite of the gibbon. J. Parasit., 58: 123-128, 1972.

Collins, W. E., Contacos, P. G., Skinner, J. C., and Guinn, E. G.: Studies on the transmission of simian malaria. V. Infection and transmission of the B strain of Plasmodium cynomolgi. J. Parasit. (In Press).

Collins, W. E., and Contacos, P. G.: Transmission of Plasmodium falciparum from monkey to monkey by the bite of infected Anopheles freeborni mosquitoes. Trans. Roy. Soc. Trop. Med. & Hyg. (In Press).

Collins, W. E., Contacos, P. G., Krotoski, W. A., and Howard, W. A.: Transmission of four Central American strains of Plasmodium vivax from monkey to man. Am. J. Trop. Med. & Hyg. (In Press).

Serial No. NIAID 133-A
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies of the Exoerythrocytic Stages of Primate Malaria

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos and William E. Collins

Other Investigators: None

Cooperating Units: Dr. Thomas M. Sodeman, University of Michigan

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	9/12			9/12
Professional	2/12			2/12
Other	7/12			7/12

Project Description:

Objectives: (1) To describe the exoerythrocytic (EE) stages of species and strains of malaria in which they have not previously been described; (2) to study the histochemical reaction of EE bodies; (3) to study the prophylactic and curative effects of antimalarial drugs on the EE stages of malaria parasites and to determine the morphologic alterations within the EE stages due to antimalarial drugs.

Methods Employed: Exoerythrocytic forms are produced in a new host by transmission through a mosquito vector from a previously infected host. This is accomplished: (1) by mosquito bite; (2) by direct inoculation of the sporozoites into the liver; or (3) by intravenous inoculation of sporozoites.

Livers are biopsied on selected days after exposure or following drug therapy. Biopsies are processed after paraffin imbedding, as frozen sections or by freeze substitution.

Major Findings: Nine liver biopsies were made in two species of non-human primates in attempts to find exoerythrocytic (EE) stages of three species of malaria parasites. Five of these were shown to contain EE bodies.

Of major significance was the finding of 6 and 8 day EE bodies of the Salvador II strain of Plasmodium vivax in Aotus trivirgatus monkeys. Only the 7 day forms had previously been observed.

Significance to Bio-medical Research and the Program of the Institute:

Since the earliest use of antimalarial chemotherapeutic agents, it has been recognized that relapses often occur in previously treated patients. It is now understood that these relapses occur because of the release of forms from the exoerythrocytic stages, these stages having been resistant to the therapy. Therefore, it is extremely important that the activity of any new candidate antimalarial drug against the exoerythrocytic stages be known. Previously, the number of host-parasite models available for the study of this drug activity in primates has been limited. The current project is providing the necessary information and techniques to enable investigators to utilize a number of additional parasite species and primate hosts to evaluate the activity of candidate drugs against pre-erythrocytic stages of the parasite.

Proposed Course of the Project: (1) The demonstration of yet undescribed exoerythrocytic stages of the different primate malarias; (2) the study of the morphologic effects of new antimalarial drugs on the EE bodies. In collaboration with Dr. T. M. Sodeman of the University of Michigan, biopsy material is being studied by electron microscopy to determine the ultrastructure of the EE bodies of different species of primate malaria.

Publications:

Sodeman, T. M., Contacos, P. G., Jumper, J. R., and Smith, C. S.: Studies of the exoerythrocytic stages of simian malaria VI. Plasmodium hylobati. J. Parasit., 58: 129-134, 1972.

Collins, W. E., Contacos, P. G., and Jumper, J. R.: Studies on the exoerythrocytic stages of simian malaria. VII. Plasmodium simiovale. J. Parasit., 58: 135-141, 1972.

Schnitzer, B., Sodeman, T. M., Mead, M. L., and Contacos, P. G.: Pitting function of the spleen in malaria: Ultrastructural observations. Science, (In Press).

Schnitzer, B., Sodeman, T. M., Mead, M. L., and Contacos, P. G.: An ultrasturctural study of the red pulp of the spleen in malaria. In manuscript.

Sodeman, T. M., Contacos, P. G., Collins, W. E., Smith, C. S., and Jumper, J. R.: Antimalarial studies on the prophylactis and radical curative activity of RC-12. Bull. Wld. Hlth. Org., (In Press).

Serial No. NIAID 133-B
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immuno-ecology of Primate Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigators: None

Cooperating Units: Central America Malaria Research Station, El Salvador

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	10/12			10/12
Professional	2/12			2/12
Other	2/12			2/12

Project Description:

Objectives: To explore the total ecology of malaria and to correlate circulating antibodies with the presence of parasites and to orient both of these factors with transmission cycles, level of endemicity, and clinical disease.

Methods Employed: Blood samples, on filter paper, are collected from human populations in areas endemic for malaria. These specimens are mailed back to the central laboratory in Chamblee, Georgia for determination of fluorescent antibody titers. The accompanying field studies vary in scope, but wherever possible the following investigations are carried out: (1) Giemsa-stained blood films of the human population for parasite rates and species identification; (2) surveys for malaria parasites in mammals other than man; and (3) studies on the anopheline fauna, including identification, seasonal distribution, biology and malaria infection rate.

Major Findings: Serologic studies in three localities in the Puntarenas area of Costa Rica indicated that malaria transmission is

probably non-existent in two of these localities (Chacarita and El Palmar) and at most at a very low level in the third (Tarcoles). Even though 48 of the 537 sera examined gave positive IFA responses, all but three were in people greater than 25 years of age.

Serologic studies for malaria in Ciricito and Cuipo on Lake Gatun in Panama have indicated that P. falciparum is the dominant malaria parasite and that transmission is still active in spite of drugs and residual insecticides. A total of 97 of 403 samples had IFA titers high enough to be considered positive. Preliminary indications are that the number of serologic positives is higher in people who said that they did not participate in the drug program than in those who said that they did participate.

In El Salvador, the number of serologic positives decreased with the altitude. In addition, there was a decided shift in the age distribution so that in populations living at altitudes of 400 feet or more above sea level, all the serologic positives were in adult males who probably acquired their malarial experience elsewhere.

Investigation of isolated outbreaks of P. falciparum and P. vivax in El Salvador indicated that many more people were involved than was indicated by slide examinations. In Jocomantique, approximately four times as many people had positive serologies as were found parasite positive.

Certain members of populations under insecticide protection were cured of their infections with chloroquine and primaquine. After a six month period, the same individuals were examined both serologically and parasitologically. Since a rise in IFA titer is indicative of recent experience with infection, it appears that this procedure may be quite useful in assessing the relative effectiveness of different antimalarial procedures in halting transmission in endemic populations.

Significance to Bio-medical Research and the Program of the Institute:

Immunological studies using the FA technique offer an opportunity to determine prior experience with malaria of populations in areas endemic for malaria. The opportunity to correlate field studies with laboratory investigations greatly expands our potential for study on the basic problems associated with human malaria.

This type of investigation gives insight into the mechanisms of the development of protective responses in populations naturally infected with malaria. Information derived from these studies will eventually enable us to predict the future course of malaria in areas of high endemicity and possibly to alert health authorities on the developing potential of epidemics.

Proposed Course of the Project: Cooperative studies will be continued with the Central America Malaria Research Station, El Salvador. These investigations will be expanded to other areas of Africa and South America as our techniques improve and the occasions arise.

Publications:

Collins, W. E., and Skinner, J. C.: The indirect Fluorescent antibody test for malaria. Am. J. Trop. Med. & Hyg., (In Press).

Serial No. NIAID 133-C
1. Parasitic Diseases
2. Primate & Human Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Fluorescent Antibody Studies in Simian and Human Malaria

Previous Serial Number: Same

Principal Investigator: William E. Collins

Other Investigator: Peter G. Contacos

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12			2/12
Professional	1/12			1/12
Other	1/12			1/12

Project Description:

Objectives: (1) To determine the development and long-term persistence of specific antibody in malaria-infected humans and monkeys; (2) to study the immunologic relationships between the different species of human and simian malarias; and (3) to determine the effect of biological and surgical immunosuppression on the development and persistence of antibody reactions.

Methods Employed: Serum samples are taken from patients and animals prior to infection and at regular intervals thereafter to determine by fluorescent antibody methods the rate of specific antibody production and the persistence and titer of antibody during the months and years following termination of the malaria infection. Animals having past malarial infection are challenged with heterologous species and strains of Plasmodium sp. and the immunologic response studied by the fluorescent antibody technique. Animals are given curative and subcurative treatments with antimalarial drugs and the effect measured using the FA technique. Animals splenectomized or otherwise surgically treated before and after infection are studied in regard to

the FA response. Sera from patients infected with malaria are studied using the specifically labeled immune globulins to determine the patterns of response to each.

Major Findings: Most of the work with IFA monkeys was done in conjunction with project 133-D which involved the determination of antibody levels subsequent to immunization and challenge with malarial antigens and parasites.

Significance to Bio-medical Research and the Program of the Institute:

There continues to be a need for additional information on the immune response of the primate host to malaria. The immunological studies using the fluorescent antibody technique offer an opportunity to measure the effect of prior experience with malaria on current clinical attack and to determine the presence of malarial experience in an endemic population. One method in elucidating the immune response is by the observation of the results of selected surgical and biological methods of suppressing immunity. The present study seeks to use these methods in exploration of the mechanisms of immunity to malaria. One of the problems which has been of interest to malariologists is that of separation of species and strains of parasite, including the question of possible identity of species infecting man and lower primates, the utilization of subspecific designations for various strains, and the possible justification of new species from strains or subspecies. The FA studies may provide additional criteria for grouping the different strains, subspecies and species of primate malaria.

Proposed Course of the Project: Further efforts will be directed toward studying the effect of immuno-suppressant drugs and anti-lymphocyte serums on the immunologic response to malaria infection. Studies will continue on the development and persistence of the antibody response in monkeys and man infected with different species of malaria and to determine the effect of multiple infection and chemotherapy on antibody response. Further efforts will be made to determine the effect of various immunization procedures on the antibody response in conjunction with project 133-D.

Publications:

Collins, W. E., Skinner, J. C., Contacos, P. G., Guinn, E. G., and Richardson, B.: The influence of splenectomy on infections of *Plasmodium fieldi* in *Macaca mulatta* monkeys. In Manuscript.

Serial No. NIAID 133-D
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1951 through June 30, 1972

Project Title: Immunological Studies on Primate Malaria

Previous Serian Number: Same

Principal Investigators: Peter G. Contacos, William E. Collins, and Wojciech A. Krotoski

Other Investigator: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	48/12			48/12
Professional	5/12			5/12
Other	43/12			43/12

Project Description:

Objectives: (1) To evaluate the possibilities for effective biologic prophylaxis against malaria; (2) to analyze the antigenic composition of various species and/or strains of simian malaria; and (3) to localize (insofar as possible) the morphological and/or antigenic unit(s) principally responsible for specific immunofluorescent reactions following naturally acquired malarial infections.

Methods Employed: For the antigenic analysis and the active immunization studies, purified parasite suspensions are obtained from monkeys at the time of peak parasitemia. Packed parasitized erythrocytes are treated with saponin. The parasites are then washed repeatedly with buffered saline. The packed parasites are either stored at -70°C or in the lyophilized state. The parasites are crushed in a Hughes tissue press and the antigenic components extracted by mixing with dilute salt solutions. Another method utilized in the preparation of antigen is to repeatedly freeze and thaw the parasitized erythrocytes. The parasitic and erythrocytic stroma are removed

by centrifugation, and the soluble malarial antigens used for various purposes. The antigens prepared are studied by gel filtration chromatography. In addition, parasitized erythrocytes treated with formalin (0.1%) are used as antigen in immunization experiments. Monkeys are actively immunized with protein extracts, with intact parasitized erythrocytes, or with intact parasites freed from the red blood cell by lysis, with fractions of hemolysates from parasitized erythrocytes, or with residue of extracted ruptured parasites. After a suitable period of time, the monkeys are challenged with malaria. Presence of antibody in the serum of the immunized animals is confirmed by indirect FA and immunoelectrophoresis. Antigenic composition of the various preparations is studied by immunoelectrophoresis. Passive immunization is studied by administering immune globulin to monkeys in a dose of 100 mg/kg. The gamma globulin is prepared from blood obtained from monkeys evidencing a degree of immunity to malaria. The ion-exchange batch technique is utilized in preparing relatively pure gamma globulin. The gamma globulin is given prior to exposing the monkeys to bites of heavily infected mosquitoes and at 15 day intervals for 3 doses. Fractionation of hemolysates from parasitized erythrocytes and aqueous extracts of parasites is accomplished by column gel filtration.

Major Findings: Nine monkeys were immunized with from 10 to 40 mg. of a heat stable (100°C) serum soluble antigen (SSA-S) of P. knowlesi given in from 2 to 4 doses. Seven of these animals survived subsequent challenge with the homologous parasite and had peak parasitemias with from 3 to 13 percent of the red blood cells infected. The control animals either died or had maximum parasitemias with greater than 22 percent of the RBCs infected. Those animals which died did so on the 8th or 9th day of patent parasitemia. There was a suggestive relationship between survival and the amount of antigen used in the immunization. Animals with the highest level of protection as measured by the degree of parasitemia also demonstrated higher IFA titers.

A subsequent experiment failed to confirm the results of this experiment. Sever monkeys were immunized with from 30 to 90 mg. of SSA-S of P. knowlesi given in from 3 to 5 doses. Only one animal survived subsequent challenge with the homologous parasite. Those animals which died did so on the 6th to the 10th day of patent parasitemia.

Therefore, there have been no significant changes from our previous observations that immunization of monkeys using a variety of immunizing materials does not completely protect from infection.

Significance to Bio-medical Research and the Program of the Institute:

Until recently the availability of reliable and effective antimalarial chemotherapy obviated the necessity for intensive immunologic studies relating primarily to biologic prophylaxis against malaria. However, with the advent of some strains of falciparum malaria resistant not only to all the accepted synthetic antimalarials but also to quinine, the need for such studies became obvious. The effectiveness with which passive or active immunization will protect monkeys against infection, not only to the homologous malaria parasite but more especially to the heterologous parasite, should indicate

the feasibility of effective biologic prophylaxis against malaria in man.

Proposed Course of the Project: The studies will be continued with further emphasis on separation of antigenic fractions by means of column chromatography. These individual fractions will then be tested for their immunizing ability against malaria. Emphasis will continue to be placed on serum soluble antigens as immunizing agents as well as immunization by irradiated sporozoites.

Serial No. NIAID 133-E
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic Studies on the Sporozoites of Primate Malarias

Previous Serial Number: 133-E and F.

Principal Investigators: Wojciech A. Krotoski, William E. Collins, and Peter G. Contacos

Other Investigator: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	12/12			12/12
Professional	4/12			4/12
Other	8/12			8/12

Project Description:

Objectives: To evaluate the immunological response of normally susceptible primate hosts to immunization with inactivated sporozoites in terms of (1) immunogenic potential, (2) complexity of the response, (3) effect on the parasite, (4) protection from naturally-induced infections, and (5) stage and species specificities. To evaluate in monkeys the efficacy of immunization against malaria by inoculation of irradiated sporozoites; to examine the feasibility of immunization of man against homologous and heterologous species of malaria using irradiated sporozoites.

Methods Employed: Infected mosquitoes are mechanically disrupted in formalinized solutions, and the resultant suspensions subjected to density gradient centrifugation in order to obtain sporozoite-rich fractions. These preparations are employed for inoculation into susceptible monkeys, and for subsequent testing in vitro of host response. Non-formalinized sporozoite preparations are also obtained for comparative testing.

At various intervals after immunization, host susceptibility to infection is evaluated by challenge by sporozoite inoculations. Host humoral responses are evaluated in terms of (1) effect on sporozoites in vitro, (2) fluorescent antibody responses, employing (a) sporozoites and (b) trophozoites as antigens, (3) species specificities, and (4) precipitin responses with trophozoite and/or sporozoite extracts.

Mosquitoes are infected with malaria parasites by feeding on infected monkeys. When sporozoites are present, mosquitoes are exposed to varying doses of X-irradiation. Sporozoites are inoculated into test monkeys by the bite of the X-irradiated mosquitoes or by the I.V. inoculation of irradiated sporozoites. After different regimens of immunization, the animals are challenged by the bites of mosquitoes infected with normal malaria parasites. The effect of immunization is measured by changes in prepatent period and course of the parasitemia in addition to serologic techniques. When, and if, a protective effect is shown by one or more of the immunizing procedures, attempts to show a similar protection in human volunteers will be taken under consideration.

Major Findings: Preliminary studies have demonstrated the feasibility of obtaining satisfactory numbers of sporozoites when sufficiently high degrees of mosquito infections have been attained. Sporozoite films of adequate density for FA testing have been prepared, and are now awaiting the completion of monkey immunizations. The greatest single difficulty in this study is that of procuring sufficient numbers of mosquitoes undergoing the high degree of infection desirable for maximum yield of sporozoites.

A total of four rhesus monkeys were repeatedly injected with sporozoite rich, formalinized preparations. Two of these received 6 monthly injections (multiple sites) of the total sporogonic cycle yield from at least 200 A. balabacensis mosquitoes infected 14-16 days previously with P. cynomolgi bastianellii. In spite of these immunizations, there were no significant alterations in the prepatent period of mosquito-transmitted P. cynomolgi bastianellii malaria, as compared with either normal controls, or controls injected with sporozoite-free-mosquito tissue. Similarly, there were no detectible differences in the course of parasitemia developed over a 30-day period in two animals receiving the greatest sporozoite stimulus. Although some evidence of stimulation of the recipients' immunologic systems was obtained by IFA testing, antibody titers were very low. It must be concluded that there is little or no immunogenic potential of sporozoites treated with even low concentrations (0.85%) of formaldehyde, when injected by the subcutaneous and intramuscular routes.

Significance to Bio-medical Research and the Program of the Institute:

Since successful sporozoite transfer from mosquito to vertebrate host is a theoretical weak link in the plasmodial life cycle, the development of immunologic resistance to this stage by the host is an attractive hypothetical approach to prophylaxis of malarial infection. The total lack of protection

by parenteral exposure to formalinized sporozoites indicates that the hypothetical immunogenic potential of sporozoites is not linked with formalinization. Successes attained in rodent malarias following injections o of irradiated sporozoites have lent support to this approach. Attempts to elicit a similar resistance in the rhesus monkey have been discouraging, although it must be stated that some delays in prepatent periods were obtained.

With the advent of strains of malaria, particularly P. falciparum, resistant to currently available antimalarial drugs, the urgent need for biologic prophylaxis became obvious. These studies using a monkey-malaria model are an extension of studies by other workers using the rodent-malaria system. If the efficacy and feasibility of such an immunizing procedure can be shown for primate malarias, the effect on the control and/or eradication of malaria would be highly significant.

Proposed Course of the Project: With the exception of further examination of sera obtained during the course of immunization with the formalin-treated sporozoite preparations, this phase of the project is completed.

If possible, the studies with X-irradiated sporozoites will be expanded to consider the minimum number of immunizing doses required, duration and degree of protection conferred, etc. The availability of adequate irradiation equipment will determine the feasibility of future studies.

Publications:

Krotoski, W. A., Collins, W. E., and Contacos, P. G.: Failure of formalinized sporozoites to protect against mosquito-transmitted malaria in monkeys. In Preparation. 1972.

Collins, W. E., and Contacos, P. G.: Immunization of monkeys against Plasmodium cynomolgi by X-irradiated sporozoites. Nature (In Press).

Serial No. NIAID 133-F
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunofluorescent Studies on the Early Exoerythrocytic Stages of Simian Malarias

Previous Serial Number: None

Principal Investigator: Wojciech A. Krotoski

Other Investigators: William E. Collins

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	3/12			3/12
Professional	2/12			2/12
Other	1/12			1/12

Project Description:

Objectives: To identify and describe early exoerythrocytic stages of simian malarias; to visualize, localize and describe sporozoite-exoerythrocyti schizogonic transformation as it occurs in situ in the natural host.

Methods Employed: Rhesus monkeys (Macaca mulatta) are immunized with (1) sporozoite-rich preparations (inactivated), (2) lyophilized, whole trophozoites and (3) trophozoite fractions (soluble and insoluble), both before and after species-homologous infections, in order to prepare antisera suitable for use in immunofluorescent test methods. Suitable sera are used to identify exoerythrocytic forms in tissue sections obtained at biopsy at appropriate intervals following direct intrahepatic inoculation of viable sporozoites. Adjacent sections are stained with suitable histochemical agents for morphologic identification and description.

Major Findings: Antisera of sufficient potency to detect sporozoites (in thin films) and blood stages in tissue sections by the IFA technique have

been prepared. Direct intrahepatic inoculations of P. cynomolgi bastianellii sporozoites have been accomplished and biopsies taken.

Significance to Bio-medical Research and the Program of the Institute:

Although sporozoite-cryptozoite transformations have been described in tissue culture for avian malaria, little is known of the transformation in vivo of the primate malarias where early exoerythrocytic forms still remain to be visualized. Visualization of the early forms could yield important clues as to the processes involved in the liver's essentially passive role (histopathologically speaking) in the development of malarial infections.

It is anticipated that these methods will assist not only in such an understanding, but more importantly in observing effects of causal prophylactic drugs on the early tissue stages of malaria.

Proposed Course of the Project: Only immunofluorescent detection and identification of early exoerythrocytic forms remain to be accomplished for completion of this project.

Serial No. NIAID 133-G
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Application of Specific Anti-Rhesus Immunoglobulin Sera to Immunofluorescent and Precipitin Studies in Simian Malarias

Previous Serial Number: Same

Principal Investigator: Wojciech A. Krotoski

Other Investigators: William E. Collins and Peter G. Contacos

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	3/12			3/12
Professional	1/12			1/12
Other	2/12			2/12

Project Description:

Objectives: To evaluate the immune responses of monkeys to malarial infection by means of anti-rhesus immunoglobulin-sera of sufficient specifici for use in indirect immunofluorescence and precipitin tests.

Methods Employed: Monkey sera (Macaca mulatta) are fractioned by gel-filtration on Sephadex G-200 into three main peaks. Peak I contains very high molecular weight proteins, including IgM; Peak II contains middle-range protei including IgG and IgA; and Peak III contains mostly albumin, hemoglobin, and similar-sized molecules. Peak I and Peak II proteins are refractionated after concentration on Sephadex G-25, and the contained immunoglobulins are identif by immunoelectrophoretic analysis (IEA) against pooled anti-whole rhesus blood serum and commercially available specific goat anti-human IgA, IgG and IgM sera. Fractions containing peak amounts of IgM are used for immunization of rabbits, while fractions containing peak amounts of IgG and IgA are partly separated by a stepwise (bulk) elution method from DEAE - Sephadex.

Subsequent purification, as required, is accomplished by ion-exchange chromatography. The specific immunoglobulin-enriched fractions are used for rabbit immunization; anti-sera thus obtained are absorbed by serum components present in peaks adjacent to those containing the Ig's, and are further purified by specific immunoadsorption onto cross-reacting human immunoglobulins conjugated to Sepharose. The specific anti-rhesus immunoglobulin sera are then conjugated with fluorescent dyes as needed, or used unconjugated in precipitin tests. Monkeys infected with various species of malaria are bled periodically for determination of serum changes.

Major Findings: A potent, monospecific rabbit anti-rhesus IgG reagent has been prepared. Rabbit anti-rhesus IgM serum, though obtained in a titer adequate for use in quantitative immunodiffusion tests (radial immunodiffusion), has yet to undergo final purification stages. Attempts to produce a rabbit anti-rhesus IgA serum in titers high enough to warrant purification to monospecificity have not been successful. A high-titered, specific rabbit anti-rhesus IgG fluorescein isothiocyanate conjugate (FITC) for use in fluorescent antibody tests has been prepared. Monkeys have been infected with one relapsing (P. cynomolgi bastianellii) and two non-relapsing (P. knowlesi, P. coatneyi) malarias, and sera are being collected for study.

Significance to Bio-medical Research and the Program of the Institute:

Although sufficient antigenic similarities exist between human and rhesus immunoglobulins to permit approximate assay of indirect monkey FA titers with anti-human Ig reagents, a greater sensitivity can be expected with the homologous anti-sera. The preparation of these reagents will permit the assay of monkey Ig's in indirect FA and precipitin testing in simian malarias with a greater degree of accuracy. This, in turn, will lead to a more detailed elucidation of the immune response in the monkey to infection with various species of malaria serving as a counterpart to human studies.

Proposed Course of the Project: The immune response in terms of IgM and IgG levels will be evaluated by fluorescent antibody and/or precipitating antibody techniques, and the results obtained correlated with species of parasite, degree of parasitemia and time course of infection.

Serial No. NIAID 133-H
1. Parasitic Diseases
2. Primate and Human Malaria
3. Chamblee & Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunologic Assays for Immuno-Active Fibrin/Fibrinogen Degradation Products in Human and Simian Malarias

Previous Serial Number: None

Principal Investigator: Wojciech A. Krotoski

Other Investigators: William A. Howard

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	1/12			1/12
Professional	1/12			1/12
Other				

Project Description:

Objectives: To provide sensitive immunologic assays for the detection of fibrin/fibrinogen degradation products in human and rhesus sera as indices of disseminated intravascular coagulation during human and simian malarial infections.

Methods Employed: Potent, mono-specific anti-human and anti-rhesus fibrinogen sera have been prepared by specific immunoadsorption of anti-fibrinogen antibodies from antisera to whole human or whole rhesus plasma and to purified human fibrinogen or rhesus euglobulin preparations. Immuno-adsorbents were prepared by conjugating a twice-purified (cold ether scheme) human fibrinogen preparation from outdated plasma, and a twice-purified (12% ammonium sulfate) rhesus fibrinogen preparation from fresh plasma to Sepharose 2B with CNBr. Traces of antibodies to other plasma components were totally removed by absorption with pooled, lyophilized whole serum (human), or passage over an immunoadsorbent column of whole rhesus serum conjugated to Sepharose.

On the basis of demonstrated antigenic relationships between fibrin and fibrinogen and their products of catabolism, these preparations are being employed in sensitive immunodiffusion tests for fibrin/fibrinogen degradation products in human and simian malarial infections. All coaguable substances are removed prior to testing by the addition of exogenous thrombin to each serum.

Major Findings: Tests of these reagents in immunodiffusion against corresponding purified fibrinogen preparations or plasma revealed single lines in both rhesus and human systems; these were identified as due to fibrinogen by immunoelectrophoresis. At appropriate dilutions, the anti-human reagent is capable of detecting the homologous fibrinogen at concentrations of not less than 50 μg/ml. The anti-rhesus reagent is capable of detecting the homologous fibrinogen a t concentrations of not less than 15 μg/ml.

Examination of 137 normal human sera obtained from prospective blood donors revealed an incidence of 2.9% positive for fibrinogen-related antigen after the addition of thrombin. When employed in testing sera from volunteers undergoing parasitemia of malaria, 38.9% (93/239) were positive.

Examination of 30 normal rhesus sera (prior to experimental infection) revealed 62.5% positive at 1:1, 19.4% positive at a dilution of 1:2, 5.7% positive at 1:4 and 3.3% positive at 1:8. Examination of rhesus monkeys infected with P. knowlesi or P. coatneyi did not demonstrate any increase in this incidence. (The essential equivalence of the human and rhesus systems is demonstrated by the similarity of normal subjects positive at equivalent concentrations, i.e., 2.9% positive at 50 μg/ml (human) vs. 5.7% positive at 60 μg/ml (rhesus).

Significance to Bio-medical Research and the Program of the Institute:

The potency and specificity of these reagents have permitted the evaluation of the presence of fibrin/fibrinogen degradation products as indices of disseminated intravascular clotting in both human and rhesus malarias. The addition of exogenous thrombin to each serum prior to testing permitted less equivocal results than had been obtained in several prior reports of consumption coagulopathy.

The importance of determining the presence and/or degree of disseminated intravascular coagulation in malaria is based not only on the need for further elucidation of the pathophysiology of the disease, but also on recent proposals that current, widely used treatment regimens could be improved by the addition of potentially hazardous anti-coagulation methods.

Proposed Course of the Project: Project completed.

Publications:

Krotoski, W. A., Briesch, P. E., Howard, W. A., Contacos, P. G., Collins, W. E., and Gell, L. S.: Coagulation parameters in early human malaria. In Preparation. 1972.

Howard, W. A., Krotoski, W. A., and Collins, W. E.: Study of coagulation parameters and the effect of heparin in simian malaria. In Preparation. 1972.

Serial No. NIAID 133-I
1. Parasitic Diseases
2. Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Comparative Histological and Histochemical Studies on the Sporogonous Cycle of Malaria Parasites

Previous Serial Number: 133-K

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigators: None

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	6/12			6/12
Professional	6/12			6/12
Other				

Project Description:

Objectives: To study (1) morphological or histological differences between the development of the parasite in mosquitoes showing different degrees of susceptibility to plasmodial infections; (2) histochemical demonstration of the relationship between the mosquito tissue and the parasite.

Methods Employed: Histological: Plasmodium sp. are studied in laboratory bred mosquitoes (Anopheles balabacensis balabacensis, A. maculatus and A. freeborni) fed on experimentally infected monkeys (Macaca mulatta). The sporogonic stages are demonstrated in paraffin sections of infected mosquitoes 0 through 21 days after the infective blood meal. Sections are stained with Giemsa or Hematoxylin-eosin. Histochemical: (a) the localization of nucleic acids (DNA & RNA) is determined by the conventional methods of Feulgen. Reaction and methyl-green pyronin with or without pretreatment with ribonuclease as well as the fluorescent technique using acridine orange; (b) the carbohydrate grouping is determined by the PAS stain with or without

pretreatment with diastase; acid mucopolysaccharides are identified by the PAS/alcian blue method; (c) proteins are demonstrated by the Mercuric-bromphenol and Ninhydrin methods; (d) in addition, some procedures used for the histochemical demonstration of lipids, and some hydrolytic and oxidative enzymes are applied to frozen sections of infected mosquitoes.

Major Findings: From the histological studies it appears that the early stages of the sporogonic cycle of the malaria parasite (exflagellation, fertilization and ookinete formation) proceed normally in mosquitoes showing different degrees of susceptibility. Meanwhile, the number of ookinetes which did succeed to penetrate the gut and establish themselves as young oocysts on the external wall of the stomach were comparatively lower in less susceptible mosquitoes. Furthermore, there was a marked difference in the rate of growth of developing oocysts in both groups of mosquitoes. In A. balabacensis, sporoblast formation and sporozoite differentiation were observed to occur a little earlier than in A. freeborni (less susceptible). In A. maculatus, some developing oocysts were impacted within the gut wall in an apparently intracellular position and developed only partially. Degenerative changes of oocysts, particularly in the later stages of development were characterized by the break-up of the oocyst content, the unorganized distribution of DNA and RNA and coagulation of the sporoplasm with the formation of amorphous DNA and RNA droplets. Oocysts containing such inclusions may degenerate partially or totally with the formation of vacuoles and deposition of refractile pigments on the surface. Large Feulgen-positive droplets were observed in mature oocysts in A. balabacensis which were lacking in those found in A. freeborni, which may suggest an over-production of DNA substance in oocysts growing in the former species. RNA positive reactions are (1) particularly marked in the cytoplasm of the oocysts, sporoblastoids, stomach epithelial cells, necks of the lateral lobes of the salivary glands, oocystes and nutritive cells; (2) less marked in the cytoplasm of the salivary gland cells, malpighian tubules cells; and (3) absent in muscle cells. Cytochemical tests indicated the absence of glycogen from the oocyst cytoplasm, whereas a positive reaction for polysaccharides was found mainly in the oocyst wall basement membranes and haemolymph. Evidence of acid mucopolysaccharides was demonstrated in developing and mature oocysts.

The histochemical tests indicate also three main regions of secretory activity in the salivary glands of female Anopheles mosquitoes: the central lobe, the lateral lobes and the necks of the lateral lobes. The acinus of the central lobe was histochemically distinct from the acini proper of the lateral lobes, which may be correlated with difference in their secretory activity. This may explain in part why invasion of sporozoites was sometimes restricted to the acinus of the central lobe while those of the lateral lobes of the same gland remain exclusively negative.

Significance to Bio-medical Research and the Program of the Institute:

The incidence of malaria in its many ecological environments in primarily dependent on those factors which bring a suitable mosquito vector in contact with the susceptible host. Although it has been recognized for many years that not all anophelines are capable of transmitting malaria, the reasons for this are, in many instances, unclear. These studies will not only assist in an understanding of the reasons for mosquito susceptibility to malaria, but more importantly, in what makes a species a vector.

Proposed Course of the Project: The study will be continued to investigate physiological and biochemical factors in anopheline mosquitoes susceptible to infection but with varying degrees of vector potential. Particular emphasis will be placed on other monkey-malaria systems and human malarias.

Serial No. NIAID 133-J
1. Parasitic Diseases
2. Malaria - Primate Malaria
3. Chamblee, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Gametocytocidal and Sporontocidal Effects of Some Commonly Used Antimalarial Drugs on the Sporogonic Cycle of Malaria Parasites

Previous Serial Number: None

Principal Investigators: Mohamed S. Omar and William E. Collins

Other Investigator: Peter G. Contacos

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	20/12			20/12
Professional	8/12			8/12
Other	12/12			12/12

Project Description:

Objectives: To investigate the effect of antimalarial drugs on the mosquito stages of the malaria parasites; to characterize the point of action of various antimalarials in the sporogonic cycle particularly on the early stages (microgamete production, fertilization, ookinete formation, gut penetration and encystment).

Methods Employed: The simian malaria parasite, Plasmodium cynomolgi bastianellii in rhesus monkeys is used in this study. Blood induced infections of the parasite are maintained by continuous syringe passage of infected blood at approximately two week intervals. Drugs are administered to infected monkeys in the form of solutions or suspensions via stomach tube. The drug dosages and regimens for the monkey are equivalent to those recommended for treatment of human malaria (mg/kg base body weight X3). Batches of Anopheles maculatus mosquitoes are allowed to feed on a rhesus

monkey infected with P. c. bastianellii as follows: (1) immediately before treatment, when gametocytes were numerous in the blood; (2) at various hours during the first day of treatment; and (3) at daily intervals thereafter. The behaviour of the early stages of sporogony is studied in smears made from the blood in the stomach of mosquitoes at intervals after feeding, in an attempt to demonstrate the effect and exact point of drug activity against sporogony, microgamete formation, fertilization and ookinete formation. Samples of infected mosquitoes taken two and three days after blood meals are fixed, sectioned and stained with Giemsa. Mosquito guts are examined and oocysts counted starting on day 6 after the infective blood meal. The salivary glands are examined for sporozoites starting on the 12th day following infection. Oocyst counts and percentage of mosquitoes infected before and after drug administration are taken as criteria for drug activity.

Major Findings: The sporontocidal effects of various drugs, including quinine, the 4-aminoquinolines, mepacrine and chloroquine, the 8-aminoquinoline, primaquine, and the folic acid reductase inhibitors, pyrimethamine and chlorguanide were tested against the sporogonic cycle of P. c. bastianelli in A. maculatus.

Quinine: A 75 mg/kg/day treatment for 5-7 days of quinine sulfate had little, if any, sporontocidal effect on the same day of drugging and the day after. Two days later, there appeared to be a remarkable sporontocidal activity. Oocysts observed in mosquitoes were retarded and failed to reach maturity. No gland infectious were recorded. It seems, therefore, that "broods" of mature gametocytes already present in the blood at the time of treatment persisted almost undamaged and proved infective for mosquitoes; however, those in the process of formation and maturation were adversely affected by the drug. Apparently, in the dose employed, quinine inhibits the growth of young gametocytes and renders them incapable of mosquito infectivity.

Chloroquine: Tests were carried out on the effect of chloroquine (65 mg/kg base) given within a 48 hour period. It was observed that chloroquine, which is generally accepted to have no action on the mature gametocytes of malaria parasites, had a marked gametocytocidal and sporontocidal effect on P. c. bastianellii in A. maculatus. The sporontocidal action was clearly demonstrated within the same day after initiation of drug therapy, whilst the gametocytocidal effect was evident 48 hours after the commencement of treatment.

Pyrimethamine: A single dose of pyrimethamine (3 mg/kg base) did exert a marked sporontocidal effect against P. c. bastianellii within one hour after drugging and subsequently. All gut infections in mosquitoes fed on days 1, 2, 3, and 4 after treatment consisted of small, abnormal oocysts which never reached maturity.

Chlorguanide: The arrest of sporogony following the administration of the first dose of chlorguanide (0.65 mg/kg/day X5) was observed within the first hour after treatment and subsequently. On days 1 through 4 after the beginning of drug therapy, some retarded oocysts were detected on mosquitoguts, but these were always very small (of the size of normal 3-day-old oocysts) and failed to grow any further beyond this stage. At 8 days, these oocysts could only be recognized as small chitinized black spots on the stomach wall.

Primaquine: Assessment of the sporontocidal effect of primaquine (1.95 mg/kg base as a single dose or 0.65 mg/kg base/day X5) showed that such treatment exerted a profound sporontocidal activity within 4 hours after first drug administration as evidenced by complete interruption of the sporogonic cycle in the mosquito. However, small retarded and abnormal oocysts were detected in mosquitoes that fed on the days following primaquine treatment. Although a single dose of primaquine exerted a pronounced sporontocidal and gametocytocidal effect against P. c. bastianellii, if asexual parasitemia persisted, new gametocytes were formed and normal mosquito infectivity resumed as early as 3 days after drug administration. These findings, which coincide with those of other workers, emphasize that primaquine may be relatively rapidly metabolized and single doses do not exert a long-lasting antimalarial effect.

Significance to Bio-medical Research and the Program of the Institute:

Although monkey-malaria models have been extensively used for the study of chemotherapy of experimental malaria, remarkably little information, if any, is available concerning the gametocytocidal and sporontocidal effects exerted by standard antimalarial drugs on the sporogonic cycle in the mosquito. Therefore, it was found expedient to undertake a detailed comparative study to illustrate the mode of action and effect of the commonly used antimalarial drugs in an attempt to determine whether each compound induces any visible effects on the sporogonic stages of plasmodia. Further attention should be given to the potential role of new drugs and drug combinations as gametocytocidal and sporontocidal agents particularly after the increased appearance of resistant strains of human malaria to currently available antimalarial drugs. In characterizing such compounds capable of preventing the completion of the sporogonic cycle would be of great value in malaria eradication programs.

Proposed Course of the Project: The studies will be continued to evaluate the effect of standard antimalarial drugs or drug combinations on the sporogonic stages in the mosquito vector using other monkey-malaria systems and human malaria as well. It is also anticipated that the efficacy of new antimalarial compounds known to have gametocytocidal and sporontocidal properties will be investigated.

Serial No. NIAID 133-K
1. Parasitic Diseases
2. Malaria - Primate Malaria
3. Chamblee, Georgia

PHS - NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Study of Coagulation Parameters and the Effect of Heparin in Simian Malaria

Previous Serial Number: NIAID 133-J

Principal Investigator: William A. Howard

Other Investigators: William E. Collins and Wojciech A. Krotoski

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	8/12			8/12
Professional	6/12			6/12
Other	2/12			2/12

Project Description:

Objectives: To confirm and further study the reported antimalarial effect of heparin; to determine whether a coagulopathy complicates the course of simian malaria; and if so, to determine whether heparin anti-coagulation is of therapeutic benefit in prolonging longevity.

Methods Employed: Rhesus monkeys were randomly divided into heparinized and non-heparinized groups and infected with P. knowlesi malaria by sporozoite (Phase A) and blood inoculation (Phase B). Additional animals were exposed to P. coatneyi malaria (Phase C) by sporozoite inoculation and were subsequently splenectomized on day 28. Two of the latter animals received a course of high dose heparin after their parasitemias had become well established. Hematologic and coagulation parameters as well as parasite counts were followed serially in all monkeys.

Major Findings: Heparin had no demonstrable antimalarial activity in and of itself and, in high doses, was associated with high morbidity and

mortality from hemorrhage. Impaired heparin clearance was noted with pre-terminal P. knowlesi malaria and marked coagulation abnormalities were consistently present in P. knowlesi infected monkeys as they became moribund. Minor abnormalities in some coagulation parameters appeared during the early acute rise in parasitemia in animals infected with P. coatneyi. These abnormalities were self limited, however, disappearing as the parasitemia fell secondary to the immune response. Minor coagulation abnormalities frequently reoccurred with the much higher and chronic levels of parasitemia attained after splenectomy, but were not associated with increased short term mortality. It appeared as though the severity of the coagulopathy correlated best with the rate of change in parasitemia and its consequent physiologic imbalances rather than with the absolute parasite count. Heparin, as the only mode of therapy, did not prolonged survival of animals infected with P. knowlesi malaria. Due to the behavior of fibrinogen as an acute phase reactant, a single determination of fibrinogen level may be misleading when evidence is sought to support the diagnosis of DIC.

Significance to Bio-medical Research and the Program of the Institute:

The study of coagulation parameters is seldom possible throughout the entire course of human malaria except in controlled volunteer infections. Here, however, the level and duration of parasitemia are necessarily limited and a coagulopathy is generally not demonstrable. Thus, simian malaria serves as a convenient model in an attempt to more completely elucidate the pathophysiology of disseminated intravascular coagulation noted in severe falciparum infections and to evaluate the effects of various forms of therapy.

This study confirms a previously contested report of a coagulopathy complicating the terminal stages of P. knowlesi malaria in the primate host. It serves as an extension of the previous work by defining the development of coagulation abnormalities in relation to other measurable parameters throughout the course of simian malarias.

Extrapolating the results of this study of human infections suggests that high levels of parasitemia may not necessarily be associated with a clinically significant coagulopathy. With its attendant risks, heparin therapy, if used in fulminant malaria, requires the careful prior documentation of DIC and then, should only be used as an adjunct to direct anti-malarial therapy and other supportive measures. Its use in human malaria for the purpose of improving parasite clearance has not basis. Heparin dosage must be carefully monitored in view of the probability of impaired clearance in situations where its use may be indicated.

Proposed Course of the Project: Project completed.

Publications:

Howard, W. A., Krotoski, W. A., and Collins, W. E.: Study of Coagulation Parameters and the Effect of Heparin in Simian Malaria. In Preparation.

Serial No. NIAID 134
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Pathogenesis of the malarial paroxysm

Previous Serial Number: Same

Principal Investigators: Franklin A. Neva, William A. Howard, and Richard H. Glew

Other Investigators: Louis H. Miller

Cooperating Units: Department of Justice, Bureau of Prisons, Federal Penitentiary, Atlanta, Georgia; Section on Medical Parasitology of Laboratory of Clinical Investigation; Dr. David W. Alling; Jack Levin, Department of Medicine, Johns Hopkins Hospital

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	41/12			41/12
Professional	6/12			6/12
Other	35/12			35/12

Project Description:

Objectives: (1) to define, with greater precision than has been done previously, the time relationship of parasite development to onset and subsequent elements of the malarial paroxysm and (2) to determine causative factors in the genesis of fever and other manifestations of the malarial paroxysm.

Methods Employed: Study of the time relationship of parasite development to the onset and subsequent elements of the malarial paroxysm is limited to patients with naturally occurring or induced malaria whose infections exhibit a highly synchronized cycle of parasite development and/or a well synchronized cycle of clinical signs and symptoms. Thin blood

smears are prepared throughout a selected paroxysm at 15 to 30 minute intervals while periodic blood specimens are taken for hematologic and serologic determinations, and body temperature is monitored. Subsequently, several observers independently perform parasite differential counts on each slide by recording total numbers of specified parasite forms observed per 80,000 RBCs.

Serum complement levels, assayed with a standardized 50% hemolytic endpoint technique, are measured at selected intervals early after the onset of patent malaria as well as late in the illness prior to treatment. When possible, a paroxysm during the primary attack as well as during a relapse is studied. Special effort is made to bracket well-defined fever paroxysms with complement determinations and correlate the serum complement levels with the immune status of the host, as measured by the IFA and CF techniques, and the parasite density.

Initial attempts to demonstrate a transferable pyrogen and/or substances responsible for the clinical manifestations of the malarial paroxysm entails withdrawing a total of 500 cc of blood in 1 or 2 aliquots at a specified time during a well synchronized paroxysm. The blood, withdrawn and handled using standard blood banking procedures, is reinfused in the same patient as whole blood or component fractions after he has been rendered smear negative for malaria with standard antimalarial medications. Patients are monitored closely for systemic complaints suggestive of the malarial paroxysm and for fever during the reinfusion. Plasma samples will be obtained at various intervals around the paroxysm for endotoxin-like activity by the limulus assay. All glassware, syringes and needles are processed rigorously to render them pyrogen free.

Major Findings: Standardization of the counting procedure and training of the observers has been completed. Parasite differentials are currently being performed on the several studies completed over the past 2 years and the results analyzed both statistically and in terms of other events occurring during the malarial paroxysm. Preliminary data indicate a degree of deep vascular schizogony in vivax malaria and for that reason, the rate of appearance of ring forms will serve as the best index of mature schizont rupture. Onset of fever and the earliest clinical symptoms correlate well with the appearance and/or initial increase in the numbers of ring forms. Chilling occurs well after the appearance of ring forms and during their most rapid increase in number. The maximal number of ring forms may be reached several hours after peak fever is attained.

Infusion of 250 ml aliquots of whole blood obtained during a paroxysm at a time when ring forms were 7% and 50% of their maximum numbers and stored for 4 to 4-1/2 days under blood bank conditions, was not productive of fever or symptomatology. Reinfusion of 500 ml of whole blood from another patient, obtained at a time when the number of ring forms was anticipated to be approximately 25% of maximum and subsequently stored for 3 days, was also non-eventful.

During paroxysms of the primary attack whole serum complement rose as an acute phase reactant during the initial epidsode of patent malaria, typically characterized by multiple daily fever spikes and relatively continuous symptomatology. A significant fall in the levels of C'_{50} and the C'4 fraction was consistently noted during the well synchronized paroxysm in subsequent relapses. Maximum falls in complement levels were found a few hours after peak temperature was reached with return to normal levels 24 hours later.

Significance to Bio-medical Research and the Program of the Institute:

Although a tremendous background of information has developed concerning the pathogenesis of fever following administration of pyrogens to experimental animals, the significance of these findings to the cause of fever in human infections is not at all certain. Furthermore, while fever as a consequence of malarial schizogony has been a recognized and accepted phenomenon for years, a precise definition of the sequence of events and the time periods involved is not available in the literature. With the background of experimental data now available it seems appropriate to seek information on the events and cause of fever in as time-honored and common human disease as malaria. Many of these studies can be carried out in association with other research on malaria in human volunteers which is conducted in the laboratory. The long term interest and research experience in fever as a basic feature of disease in the Laboratory of Clinical Investigation can be utilized as approaches for further study are developed. Information that may be derived from this project about the cause of fever in a human disease may be applicable to other diseaes characterized by fever.

Proposed Course of Project: Parasite differential counts in our analyses will continue. Additional attempts to demonstrate a transferable pyrogen are planned with consideration given to the stability of the pyrogenic factor. More detailed studies on complement will be undertaken as a separate project.

Serial No. NIAID 134-A
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Evaluation of Antimalarial Drugs and/or Drug Regimens

Previous Serial Number: Same

Principal Investigators: Louis H. Miller, William A. Howard and Richard H. Glew

Other Investigators: None

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	9/12			9/12
Professional	4/12			4/12
Other	5/12			5/12

Project Description:

Objectives: To evaluate standard antimalarial agents and/or new drugs known to have antimalarial activity and to develop acceptable regimens for their use against human malaria.

Methods Employed: Healthy inmate volunteers at the U.S. Penitentiary, Atlanta, Georgia, are screened before they are admitted to the project. Volunteers are under 24-hour care and supervision of the resident investigators. For the evaluation of chemotherapeutic agents against malaria, volunteers are exposed to infection by the bites of infected mosquitoes. For studies on the prophylactic and/or suppressive effect of drugs, volunteers are medicated before exposure to infection. For studies on the therapeutic and/or radical curative effects of drug, volunteers do not receive drugs until patent infection is demonstrated. Volunteers are observed for a minimum of six months although follow-up has been extended at times for up to 18 months.

Major Findings: Antimalarial Efficacy of Lincomycin Analogs: The antimalarial activity of Cleocin® (7-chlorolincomycin - clindamycin) has been tested against three species of human malaria using a standard treatment regimen of 450 mg orally every 6 hours for 7 consecutive days (28 doses total). In five cases of vivax malaria, parasite clearance occurred in 96 to 168 hours after initiation of therapy while clinical manifestations (including fever) cleared in 48 to 102 hours. Two infections with ovale malaria also were treated with the following results: parasite clearance of 120 to 168 hours and disappearance of clinical symptoms in 120 to 144 hours. The standard clindamycin regimen was terminated in two P. falciparum infections after 9 and 13 doses when parasite counts rose suddenly to 9700/mm^3 and 4750/mm^3, respectively. A third patient with P. falciparum infection completed the full course of therapy and demonstrated no evidence of recrudescence. The combination of quinine sulfate, 650 mg orally every 8 hours for 9 doses followed immediately by the standard 7 day course of clindamycin proved curative in three non-immune volunteers infected with RI chloroquine-resistant falciparum malaria (maximum parasitemia range from 1650/mm^3 to 11000/mm^3).

Clindamycin is "slow acting" when used against infections of P. vivax, P. falciparum, and P. ovale. Although this characteristic limits the usefulness of the drug as a single therapeutic agent in falciparum malaria, clindamycin-quinine combination therapy appears to hold considerable promise in the treatment of drug-resistant strains of P. falciparum.

Significance to Bio-medical Research and the Program of the Institute:

The need for additional chemotherapeutic methods and drugs against malaria is becoming more acute with the increased appearance of strains of falciparum malaria resistant not only to all commonly used synthetic drugs but also to quinine.

Proposed Course of the Project: To study the therapeutic potential of clindamycin against selected strains of falciparum malaria (RII AND RIII chloroquine-resistant P. falciparum) when administered concomitantly with quinine in various dosage regimens.

Serial No. NIAID 134-B
1. Parasitic Diseases
2. Primate Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Characterization of New Isolates of Human Malaria.

Previous Serial Number: Same

Principal Investigators: Peter G. Contacos, William A. Howard, Louis H. Miller and Richard H. Glew

Other Investigators: Franklin A. Neva and William E. Collins

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	27/12			27/12
Professional	7/12			7/12
Other	20/12			20/12

Project Description:

Objectives: To study the response to drugs of new isolates of P. falciparum and P. vivax and the relapse patterns of P. vivax in man. Particular emphasis has been placed on relapse pattern of newly isolated strains of Central American P. vivax after treatment of acute attacks with schizonticidal drugs. The new human malarial isolates will also be studied in non-human primates, mainly the night monkey (Aotus trivirgatus).

Methods Employed: Healthy inmate volunteers at the U. S. Penitentiary, Atlanta, Georgia, are screened before they are admitted to the project. They are exposed to infection by the bites of infected mosquitoes. They submit freely to necessary laboratory procedures and receive antimalarial or other medications only as prescribed by the investigators. Participation in any one project may range for 6 - 18 months depending on the nature of the project. Standard suppressive and curative regimens of antimalarial treatment are administered and the patient observed for immediate clinical and parasitological response and for longer periods of time to determine effect of the

infection on the formed elements of the blood and on coagulation and other blood factors. Serum is obtained at various intervals and frozen for subsequent evaluation of antibody titers. For the latter tests, fluorescent antibody determination and separation of serum protein by various electrophoretic methods are utilized.

Major Findings:

1. Studies on human malaria in human volunteers.

A. Nica strain: This strain from Nicaragua was isolated in an owl monkey. To date, 9 volunteers have been exposed by bites of infected mosquitoes. Those volunteers receiving a high sporozoite inoculum (as judged by total mosquito gland ratings of 63+ to 72+) had a prepatent period of 9.75 ± 1.0 days with early primary (85-94 days) and frequent (every 20-21 days) subsequent relapses. These findings are in contrast to the 12.8 ± 1.7 day prepatent period with late primary relapses (>150 days) in those volunteers receiving low sporozoite inoculum (4+ to 8+). Insufficient data is available to permit comment on subsequent relapse activity in this latter group. The strain demonstrates sensitivity to chloroquine, quinine, and primaquine.

B. Chesson strain: In order to evaluate the possibility of alteration of relapse characteristics of this strain secondary to several years of serial primate passage, two volunteers received large sporozoite inocula (49+ to 57+) by the bites of infected mosquitoes. Their short prepatent periods (11 and 12 days) and frequent relapse pattern (every 19 days) was consistent with the earlier characterization studies.

Evaluation of a five day primaquine regimen consisting of 15 mg of the base daily was undertaken in four additional volunteers. Although one volunteer did relapse after this regimen, intrepretation of the results is hampered by the tendency for irregular and infrequent relapses in these patients subsequent to the lower sporozoite inocula they received.

11. Studies on human malaria in non-human primates.

In our continuing studies concerned with the experimental infection of New World monkeys with human malaria, the major findings for the year can be summarized as follows:

1. Transmission to an owl monkey (Aotus trivirgatus) of the Cambodian 1 strain of P. falciparum by the bites of infected A. freeborni mosquitoes.

2. Infection of owl monkeys with two new isolated of P. vivax (South Vietnam IV and Pakchong). The origin of the Pakchong strain is Thailand.

3. Transmission of the Chesson and Nica strains of P. vivax from owl monkey back to man by the bites of infected mosquitoes.

4. The Salvador 11 strain of P. vivax was transmitted from owl monkey to owl monkey by the bites of infected A. freeborni mosquitoes.

5. The Ehesson strain of P. vivax was transmitted to owl monkey from man by the bites of A. maculatus mosquitoes.

Significance to Biomedical Research and the Program of the Institute:

Malaria remains one of the major world-wide causes of morbidity and mortality. In recent years, reports of Americans returning to this country and developing malaria with onset in this country are also increasing. The imported malarias have the potential for infecting local anopheline mosquitoes, and it is necessary to study some of these imported strains of malaria to determine their clinical manifestations, the ease with which they can be transmittedby anopheles from the U. S., and their sensitivity to the standard antimalarial drugs.

Study of relapse patterns of Central American P. vivax in human volunteers has been of help to the eradication program in Central America. It has been noted in areas of Central America that the incidence of vivax malaria increases during the dry season, approximately 6 months after the main malaria transmission season. Although no anopheles vectors were found at that time, it was possible that the vectors were missed by the eradication research team. From the data on relapse patterns in volunteers, it appears that the increased incidence during the dry season is caused by relapses and not renewed transmission.

The fact that small non-human primates can be infected with human malaria could permit studies of the biology and chemotherapy of human malaria. However, the problems of adapting the human strains to Aotus have been formidable. Except on rare occasions it is not possible to infect Aotus monkeys with viable P. falciparum sporozoites. Therefore the strains is transmitted by blood subpassage and soon looses the ability to produce gametocytes and infect mosquitoes. Although it has been easier to transmit P. vivax from monkey to monkey by mosquitoes, the biology is far from normal, and the infection rate of monkeys by mosquitoes is about 50%.

Proposed Course of the Project: To obtain new isolates of P. vivax from Central America to define relapse patterns. To determine to effect of 5 days of primaquine 15 mg/day on relapses of Central American P. vivax. The drug resistant strains of P. falciparum from Central and South America will be characterized as to their sensitivity to schzonticidal drugs. Transmission of new strains of human malaria will be attempted in the night monkey.

Publications:

Krotoski, W. A.: Hepatitis in prisoner blood donors. Correspondence, New England J. Med. 286: 159, 1972.

Contacos, P. G., Collins, W. E., Jeffery, G. M. Krotoski, W. A. and Howard, W. A.: Studies on the characterization of Plasmodium vivax strains from Central America. Amer. J. Trop. Med. & Hyg. (In Press).

Howard, W. A., Krotoski, W. A., Slonim, M. S. and Contacos, P. G.: Spontaneous splenic rupture in experimental vivax malaria. Mil. Med. (In Press).

Collins, W. E., Contacos, P. G., Krotoski, W. A. and Howard, W. A.: Transmission of four Central American strains of Plasmodium vivax from monkey to man. J. Parasit. (In Press).

Chin, W. and Coatney, G. R.: Relapse activity in sporozoite-induced infections with a West African strain of Plasmodium ovale. Amer. J. Trop. Med. & Hyg. 20: 825-827, 1971.

Serial No. NIAID 134-C
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cardiopulmonary Changes in Malaria Human Volunteers

Previous Serial Number: None

Principal Investigators: Richard H. Glew and Louis H. Miller

Other Investigator: William A. Howard

Cooperating Units: Department of Justice, Bureau of Prisons

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	2/12			2/12
Professional	2/12			2/12
Other	0/12			0/12

Project Description:

Objectives: This study is designed to evaluate possible changes in arterial oxygen saturation during the malarial paroxysm and to elucidate possible pathophysiologic mechanisms responsible for these changes.

Methods Employed: Volunteers infected with P. vivax and P. falciparum by the bites of infected mosquitoes are studied. Routine spirometry is performed prior to study and sequentially during the paroxysm under study. On each volunteer, several arterial samples are drawn during the paroxysm under study; in particular, blood gas determinations are made during the chilling and flushing phases. When hypoxia is detected, 100% oxygen is administered for 10 minutes to the volunteer by face mask via non-rebreathing valve to distinguish between hypoxia secondary to arterial venous shunting and other causes (diffusion defect or ventilation perfusion imbalance). Frequent chest x-rays and physical examination are made throughout the study.

Major Findings: Preliminary studies on four patients in whom blood gas studies were performed at a clinical laboratory off site revealed mildly decreased arterial PO_2 and normal to low arterial PCO_2, indicating hypoxia without hypoventilation.

Significance to Bio-medical Research and the Program of the Institute:

Respiratory complaints without obvious etiology have been described in the older malaria literature and have been noted to occur on occasion in human volunteers experimentally infected with malaria. In addition, severe cardiopulmonary changes, manifested as pulmonary edema, are known to appear in severe cases of Plasmodium falciparum malaria. Evaluation of blood gases during malria may provide information about possible pathophysiology of cardiopulmonary function in malaria.

Proposed Course of the Project: After unavoidable delay, an 1L-113 blood gas apparatus has been obtained and the proposed studies should commence in the near future. As soon as the blood gas apparatus is operational, blood gas determinations will be begun on volunteers as their infections become patent.

Serial No. NIAID 134-D
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on Blood Coagulation in Human Malaria

Previous Serial Number: None

Principal Investigator: Wojciech A. Krotoski

Other Investigators: William A. Howard, Peter G. Contacos and William E. Collins

Cooperating Units: Peter E. Briesch, Coeur d'Alene, Idaho

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	1/12			1/12
Professional	1/12			1/12
Other	0/12			0/12

Project Description:

Objectives: To evaluate alterations in coagulation parameters and the incidence and severity of disseminated intravascular coagulation in human volunteers infected with several species of malaria.

Methods Employed: A number of human volunteers were infected with Plasmodium vivax, P. ovale, P. falciparum or P. malariae. Blood was drawn for coagulation studies for several days before the development of patent parasitemia, then daily during patency and several times after reversion to smear negativity. The following parameters were evaluated: hematocrit, reticulocyte count, leukocyte and platelet counts, prothrombin time, partial thromboplastine time, thrombin clotting time, euglobulin lysis time and plasma fibrinogen concentration. In addition, a semi-quantitative estimation of serum fibrinogen/fibrin degradation products (F.D.P.) was performed by immuno-diffusion in agar gel, employing a monospecific anti-fibrinogen serum.

Major Findings: The most consistent finding was the development of thrombocytopenia in a significant number of volunteers. This was seen in all of the patients with falciparum malaria, and in about two-thirds of those with vivax, ovale and quartan malarias. However, some degree of platelet depression was seen in all subjects, the nadir of the platelet count coinciding roughly with the peak of parasitemia. Levels began to rise soon after clearance of parasites from the circulation, usually reaching normal values within about two weeks.

Although prothrombin times and PTTs were in no case prolonged to clinically significant levels, some prolongations did occur with falciparum (PT & PTT) and ovale (PT) malarias when these groups were compared by day of parasitemia. No significant changes were noted with either vivax or malariae infections. No prolongation of thrombin clotting times were detected in any of the malarias studied; in fact, trends were suggestive of a very slight shortening of clotting time, although these are of uncertain significance. Determinations of plasma fibrinogen levels suggested minor increases in this factor during the course of all the malarias. Euglobulin lysis times were significantly prolonged from baseline levels, the most rapid development of this change occurring in falciparum and ovale malarias.

Fibrin/fibrinogen degradation products, detected as fibrinogen-related antigen (F-R. Ag), were present at levels of 50 μ/ml or greater (fibrinogen equivalent) in 27% (11/41) of sera tested during P. vivax infection, 35% (16/46) of sera tested during P. ovale parasitemia, 34% (25/73) during P. falciparum and 44% (35/79) during P. malariae infections. A single volunteer with P. knowlesi parasitemia had 60% (6/10) of sera positive for F-R.Ag. In contrast, only 2.9% of 137 normal sera (blood donors) tested were positive by this method. The appearance of F-R. Ag did not correlate well with any of the coagulation parameters studied, nor was it related to degree of parasitemia. Positive results were obtained at parasitemias from less than 50 per cu.mm. to 70,000 per cu.mm.; conversely, tests were negative in several instances where parasitemia exceeded the latter level. Negative results could be obtained after several previously positive results, to be followed by a positive test once again during the fall of parasitemia after specific treatment. In no instance were manifestations of cerebral involvement detected in any volunteer; renal function, as evidenced by normal serum creatinines around parasitemia peaks, remained normal.

Significance to Bio-medical Research and the Program of the Institute:

The original report of disseminated intravascular coagulation as an accompaniment of falciparum malaria by Dennis et al was proposed as the pathogenetic basis for cerebral malaria in P. falciparum infections, as well as a rationale for the use of anticoagulant therapy where this complication threatened. Subsequent reports of higher levels of F-R. Ag in cases of cerebral malaria than in cases where no cerebral manifestations were found (Reid and Nkrumah; Jaroonvesema) also could be used as support for treatment with heparin. However, cause and effect of F.D.P. generation

in vivo are easily confused, while heparin therapy is potentially hazardous in instances where cerebral hemorrhage could follow compromise of cerebral circulation. For these reasons the mere presence of F.D.P. in the absence of additional evidence of problematic fibrin deposition is a dubious basis for heparin anti-coagulation.

This study, while not giving definitive answers to the question of the role of disseminated intravascular coagulation in the etiology of cerebral malaria, does permit the following conclusions: (1) a basic similarity exists in the effects on blood coagulation of each of the malarias studied, (2) *P. falciparum*, and possibly *P. ovale*, may somehow have a slightly greater influence on the prolongation of prothrombin time and/or partial thromboplastin time than the other human malarias, (3) fibrin/fibrinogen degradation products (measured as fibrinogen-related antigen) were present in a proportion of all species of malarias studied, and (4) heparin anticoagulation should not be instituted solely on evidence of the presence of fibrin/fibrinogen degradation products; additional indications of significant disseminated intravascular coagulation must be present to justify its use.

Proposed Course of the Project: Several additional volunteers infected with *P. vivax* will be studied in order to increase the sampling for this malarial species.

Serial No. NIAID 134-E
1. Parasitic Diseases
2. Malaria - Human Malaria
3. Atlanta, Georgia

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Human Malaria and the Biologic False Positive Test for Syphilis

Previous Serial Number: None

Principal Investigator: Wojciech A. Krotoski

Other Investigators: Peter G. Contacos and William A. Howard

Cooperating Units: Arnold F. Schroeter and Donald Prinz; VDRL Research Division, CDC, Atlanta, Georgia

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Worker	Total
Total	1/12			1/12
Professional	1/12			1/12
Other	0/12			0/12

Project Description:

Objectives: To determine the incidence of biologic false positive tests for syphilis in human malaria as detected by current serologic-test-for-syphilis techniques.

Methods Employed: Serum specimens obtained at frequent intervals before, during and after malarial infection with each of the four human species were tested by standard (VDRL) flocculation and FTA-Abs techniques, and the incidence of biologic false positive results determined. An attempt was also made to determine the etiology of any biologic false positive reactions that occur.

Major Findings: Of 51 male volunteers initially negative by both the VDRL and FTA-Abs tests, only two developed unequivocal biologic false positive (BFP) flocculation tests (FTA-Abs negative) following malarial infection; both were Negroes. One, with falciparum malaria, was first noted to have a reactive

test 5 days after onset of parasitemia; the initial peak titer of 1:128 gradually declined to a titer of 1:2 on the 45th day after onset (a total of seven days of parasitemia had been experienced), and a serum drawn 18 weeks after onset was non-reactive. The other, with vivax malaria, was first reactive 8 days after onset of parasitemia; his initial titer of 1:2 rose to 1:4 two days later, then declined to weakly reactive in undiluted serum on the 72nd day after onset (total of nine days of parasitemia). During a relapse 7 months later his serum was again weakly reactive at 1:1 on the 11th day after onset of patency, but was non-reactive on the 25th day after onset (a total of 10 days of parasitemia had been experienced during the relapse). Both volunteers denied any possible exposure to syphilis during the test period.

Although BFP reactivity of the first volunteer's serum (with a reactivity of 1:64) was abolished by exposure to mercaptoethanol followed by overnight dialysis in the cold vs. PBS, dialysis alone also abolished reactivity. Attempts at characterization of the reactive principle by gel filtration on Sephadex were unsuccessful, since no fractions exhibited reactivity following this procedure.

Significance to Bio-medical Research and the Program of the Institute:

Reports of BFP flocculation reactions in serologic tests for syphilis in association with malaria range from 100% in 1952 to 10.7% in 1970. Our findings are in considerable disagreement with these figures, since the incidence in our controlled series was only 3.9%. This discrepancy is probably due to improvements in the performance of the VDRL test, possibly in the nature of the antigen employed.

In view of the early onset of BFP reactivity, it would be tempting to consider the concomitant rise in IgM due to malaria as responsible. However, both the lability of the reactive principle and its persistence over an extended period of time would tend to argue against this hypothesis. Nevertheless, the substance or substances responsible have yet to be identified.

Proposed Course of the Project: This project is completed.

Serial No. NIAID 67C

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on arboviruses

Previous Serial Number: Same

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Duane Gubler, Dr. Timothy Kuberski, Dr. Lloyd Rozeboom, Dr. Jean-Paul Moreau, Dr. Jean Lagraulet, Dr. Jacques Saugrain, Dr. Guy Loison

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; Institut de Recherches Medicales, Papeete, Tahiti; South Pacific Commission, Noumea, New Caledonia.

Man Years:	NIAID staff	Guest Worker
Total:	21/12	10/12
Professional:	21/12	10/12
Other:	0/12	

Project Description:

Objectives

1. To study the epidemiology of arboviruses in the simple ecologic environment of Pacific Islands.

2. To study the relative susceptibility of various species of mosquitoes to infection with dengue viruses.

3. To improve laboratory methods for the detection of dengue viruses and their antibodies.

Methods Employed

Epidemics of arbovirus disease are studied as they occur on Pacific islands. During the year, investigations were carried out on two outbreaks of dengue, one in French Polynesia and the other in New Caledonia.

Also, studies were continued on replication of dengue viruses after intrathoracic inoculation or feeding of mosquitoes, and on the parameters of plaque production by these viruses in LLC-MK_2 cells.

Major Findings

Tahiti dengue outbreak

An extensive outbreak of dengue was recognized on Tahiti in June, 1971 and the disease eventually affected more than one-half the population and spread to neighboring islands in the Society group. Although the outbreak had the usual devastating affect on the life and economy of the island, its most unusual feature was the coincident occurrence of a relatively high prevalence of hemorrhagic phenomena. The most common hemorrhagic manifestations were gastrointestinal bleeding in adults and hematuria in children. At least three deaths appeared to have been caused by dengue with hemorrhagic complications, and although ideally spaced serum samples were not available from most hemorrhagic cases, the sum of the evidence strongly suggested that the Tahiti outbreak represented the first occurrence of "dengue hemorrhagic fever" outside of Southeast Asia since this syndrome was recognized in 1954.

It was of considerable interest that, with one exception, all patients with severe hemorrhagic manifestations that were studied had secondary-type antibody responses. One virus strain from the epidemic has been identified serologically as dengue type 2 and the behavior in cell culture of more than 50 other strains isolated suggest that they also belong to this serotype. It is pertinent to note that this is the second major outbreak of dengue in Tahiti in recent years - the first (due to dengue type 3) having occurred in 1964-65.

New Caledonia dengue outbreak

An extensive outbreak of dengue was recognized on New Caledonia in January, 1972 and to date has affected more than one-half the population of the principal city, Noumea. In view of the findings from Tahiti, this outbreak was of considerable interest since it was probably caused by the same strain of virus. (There is considerable interchange of population between these two french-controlled islands even though they are more than 2000 miles apart.) However, unlike Tahiti, New Caledonia had had no dengue since 1944. (Both islands had had dengue type 1 outbreaks in that year.) Although investigations are still in progress, it is of interest to note that the only severe (and fatal) hemorrhagic case that has been observed

was a Tahitian immigrant aged 19 who had a secondary antibody response (and hence had been infected with dengue type 3 in Tahiti before moving to New Caledonia). This particular patient had a syndrome typical of the dengue shock syndrome seen in Southeast Asia.

Comparative susceptibility of different mosquito species to dengue

High-titered pools of dengue virus of all four prototype strains were successfully prepared without passage in either cell cultures or mice by inoculating susceptible species of mosquitoes intrathoracically with human or monkey serum. With one exception, no differences in comparative susceptibility to infection were found when these virus pools were titrated by the intrathoracic route in five species (aegypti, albopictus, polynesiensis, pseudoscutellaris, and scutellaris)of the subgenus Stegomyia of the genus Aedes. (Aedes scutellaris appeared somewhat less susceptible to infection with dengue type 3 than were the other four speces.) In contrast, a species of Culex and one of Armigeres were generally resistant to infection with the same viruses by the same route of inoculation.

After considerable initial difficulty, the seven species of mosquitoes mentioned above were successfully exposed to dengue types 1 and 2 by ingestion by offering them virus mixed with serum, erythrocytes, and sugar solution. Again, all species of Stegomyia became infected whereas Culex and Armigeres did not. In contrast to the inoculated mosquitoes, all exposed specimens of a susceptible species did not become infected by ingestion - suggesting individual variation in susceptibility. Surprisingly, a higher percentage of apparently resistant individuals was found in the case of Aedes aegypti than for the other Stegomyias - which otherwise had comparable rates of infection.

Improvement in dengue laboratory techniques

Extensive experimentation using intrathoracic inoculation of Aedes albopictus confirmed the usefulness of this technique for the detection and assay of dengue viruses, and its superiority to other currently used techniques (primate and mosquito cell cultures and suckling mice). For example, sera collected within three days of onset from humans naturally infected with dengue viruses usually had at least 10^6 ID_{50} per ml. when titrated in mosquitoes and infected mosquitoes usually contained 10^6 ID_{50} per individual. These titers are higher by several orders of magnitude than those obtained when the same materials are assayed in cell cultures or mice. Using mosquito inoculation, dengue viruses were successfully isolated from numerous samples of human and monkey sera which had failed to yield virus when tested in cell cultures.

It was found that male mosquitoes were just as susceptible as females - a finding which eliminated the danger of accidental transmission should an infected mosquito escape. It was also found that mosquitoes of various ages

were equally susceptible and that the extrinsic incubation period could be shortened to as little as four days (at 32° C).

Mosquitoes infected with each of the four prototype viruses yielded dengue complement-fixing antigen - suggesting the possibility of typing dengue isolates by a simple CF technique directly from first passage experimentally infected mosquitoes.

Since it is still necessary to test mosquitoes for plaque production in LLC-MK_2 cells to determine if they are infected, a successful attempt was made to simplify the procedure by adapting it to a multi-welled plastic plate. The new procedure saves considerable time and reagents.

Significance to Biomedical Research and the Program of the Institute

Dengue viruses are increasing in significance as a cause of morbidity and mortality in Southeast Asia and Pacific Islands - probably because of rapidly increasing urbanization and travel in that geographic area. Mosquito vectors of these viruses are ubiquitous in the same area, including many islands which belong to, or which are controlled by, the United States. Obviously, further understanding of the epidemiology and vectors of dengue would be of value.

Despite the importance of dengue viruses as causes of human disease, they have been among the most difficult to work with in the laboratory. The intrathoracic inoculation technique appears to have provided the sensitive laboratory tool which has heretofore been lacking.

Other arboviruses also are important causes of human disease in both temperate and tropical areas. Aside from dengue, studies on Pacific islands should yield information about such agents not easily obtainable in more complex ecologic settings.

Proposed Course of the Project

Considerable effort during the coming year will be devoted to working up the large amount of material collected during the Tahiti and New Caledonia outbreaks.

Work will continue on the intrathoracic inoculation technique to determine if its use can improve the presently unsatisfactory dengue neutralization test and if hemagglutinating antigens can be prepared from mosquitoes. Also, studies on dengue CF antigen in mosquitoes will continue to determine if individual infected mosquitoes can be detected by CF alone, thus bypassing entirely the need for cell cultures.

It is also planned to undertake quantitative studies of dengue viremias and their relationship to mosquito infection in cynomolgus monkeys, chimpanzees, and, possibly, human volunteers.

Of course, should an outbreak of arbovirus disease occur on a suitable island, new field studies would be undertaken. Because of their relative isolation, the frequency of infection on such islands is much less than in continental areas and opportunities for study are largely a matter of chance.

Honors and Awards

Dr. Rosen received the Meritorious Service Medal of the U.S. Public Health Service; was elected to the Executive Committee of the American Committee on Arthropod-borne viruses; and was appointed a Temporary Adviser on dengue by the World Health Organization.

He continued to serve as a Councilor of the American Society of Tropical Medicine and Hyeiene; as a member of the Virus Diseases Panel, U.S.-Japan Cooperative Medical Science Program; as an Associate Member, Commission on Viral Infections, Armed Forces Epidemiological Board; as a member of the Dengue Task Force of the same Commission; as a member of the Research Resources Committee, NIAID; as a member of the ad hoc Enterovirus Advisory Group, NIAID:; as Clinical Professor of Tropical Medicine and Medical Microbiology, University of Hawaii School of Medicine; and as a member of the Affiliate Graduate Faculty, University of Hawaii School of Public Health.

Dr. Rosen organized a conference on the "Pathogenesis of Arboviral Infections" held in Honolulu in September, 1971 under the joint sponsorship of the U.S. and Japanese Viral Diseases Panels of the U.S. - Japan Cooperative Medical Science Program and was asked (and agreed) to serve as co-chairman of a workshop on the "Biologic Control of Mosquitoes" to be held under the joint sponsorship of the U.S. Viral and Parasitic Diseases Panels of the same Program.

Publications:

Saugrain, J., Rosen, L., Outin-Fabre, D., and Moreau, J.P.: Une récent épidémie d'arbovirose du type dengue a Tahiti comparaisons avec l'épidémie de 1964. Bull. Soc. Path. Exot. 63:636-642, 1970.

Dean, A. G. and Jones, T. C.: Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. I. Clinical and epidemiologic investigations of the acute illness. Amer. J. Epidem. 95:111-127, 1972

Jones, T. C., Dean, A. G., and Parker G. W.: Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. II. Malabsorption following the acute illness. Amer. J. Epidem. 95:128-139, 1972.

Dean, A. G., Couch, R. B., Jones, T. S. and Douglas, R. G., Jr.: Seasonal gastroenteritis and malabsorption at an American military base in the Philippines. III Microbiologic investigations and human volunteer experiments. Amer. J. Epidem. (in press)

Dean, A. G., Ching, Y-C., Williams, R. G. and Harden, L.B.: An infant mouse test for E. coli enterotoxin and a study of pediatric diarrhea in Honolulu. J. Infect. Dis. (in press)

Rosen, L.: Reoviruses. In Standardized Methods for Veterinary Microbiology, to be published by the National Academy of Sciences (in press).

Rosen, L.: Reoviruses. In Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man - 6th Edition, (in press).

SERIAL NO. NIAID 67D

1. LABORATORY OF PARASITIC DISEASES
2. PACIFIC RESEARCH SECTION
3. HONOLULU, HAWAII

PHS-NIH
INDIVIDUAL PROJECT REPORT
JULY 1, 1971 THROUGH JUNE 30, 1972

PROJECT TITLE: STUDIES ON THE NATURAL HISTORY OF TOXOPLASMA GONDII, THE MECHANISM OF ITS TRANSMISSION TO MAN, AND ITS PUBLIC HEALTH IMPORTANCE IN THE PACIFIC AREA.

PREVIOUS SERIAL NUMBER: SAME

PRINCIPAL INVESTIGATOR: DR. GORDON D. WALLACE

OTHER INVESTIGATORS: DR. ALISTAIR PHILIP, DR. D. CARLETON GAJDUSEK, DR. VINCENT ZIGAS, AND MR. AND MRS. MAC MARSHALL

COOPERATING UNITS: DEPARTMENT OF PEDIATRICS, UNIVERSITY OF HAWAII SCHOOL OF MEDICINE; STUDY OF CHILD GROWTH AND DEVELOPMENT AND DISEASE PATTERNS IN PRIMITIVE CULTURES, NINDS; PUBLIC HEALTH DEPARTMENT OF THE TERRITORY OF PAPUA AND NEW GUINEA; DEPARTMENT OF ANTHROPOLOGY, UNIVERSITY OF WASHINGTON; PALO ALTO RESEARCH FOUNDATION; HAWAII STATE DEPARTMENT OF PUBLIC HEALTH; BISHOP MUSEUM.

MAN YEARS: NIAID STAFF

TOTAL:	10/12
PROFESSIONAL:	10/12
OTHER:	0/12

PROJECT DESCRIPTION:

OBJECTIVES

TO DETERMINE THE METHOD, OR METHODS, BY WHICH MAN BECOMES INFECTED WITH T. GONDII AND THE PUBLIC HEALTH IMPORTANCE OF THIS PARASITE, PARTICULARLY IN UNDEVELOPED AREAS WITH A HIGH PREVALENCE OF HUMAN INFECTION.

METHODS EMPLOYED

LIFE HISTORY AND EPIDEMIOLOGY. TAKING ADVANTAGE OF UNIQUE EPIDEMIOLOGIC SITUATIONS THAT OCCUR ON PACIFIC ISLANDS, ALONG WITH LABORATORY EXPERIMENTATION, THE FOLLOWING WORKING HYPOTHESES WERE TESTED: 1) CATS ARE THE DEFINITIVE HOSTS OF T. GONDII; 2) CERTAIN SMALL VERTEBRATE ANIMALS, NORMALLY THE

PREY OF CATS, ARE OBLIGATORY INTERMEDIATE HOSTS; 3) IN THE PACIFIC AREA, MAN USUALLY BECOMES INFECTED WITH TOXOPLASMA BY INGESTING OOCYSTS; 4) INVERTEBRATE TRANSPORT HOSTS ARE INVOLVED IN MAINTAINING THE LIFE CYCLE OF THE PARASITE AND ITS TRANSMISSION TO MAN.

EXPERIMENTATION INCLUDED OBSERVATIONS ON THE SEROLOGIC AND IMMUNOLOGIC RESPONSE AND OOCYST PRODUCTION IN CATS EXPOSED TO ONE OF THE THREE STAGES (TROPHOZOITE, CYST, OOCYST) OF TOXOPLASMA. OBSERVATIONS WERE ALSO MADE ON THE COURSE OF INFECTION FOLLOWING EXPOSURE TO OOCYSTS IN SEVERAL SPECIES OF SMALL VERTEBRATES INCLUDING TWO SPECIES OF WILD RATS (R. RATTUS AND R. EXULANS), COMMON ON MOST PACIFIC ISLANDS; FRINGILLID BIRDS (SPARROWS AND CARDINALS); AND GECKOS, A FAMILY OF LIZARDS THAT ARE KNOWN TO BE INTERMEDIATE HOSTS OF A CAT HELMINTH. EXPERIMENTS WERE CONDUCTED ON COCKROACHES TO DETERMINE IF THESE INSECTS HARBOR AND EXCRETE VIABLE TOXOPLASMA OOCYSTS AFTER FEEDING ON INFECTIOUS CAT FECES.

FIELD STUDIES INCLUDED COMPLETION OF THE STRAY CAT PROJECT INITIATED ON OAHU IN SEPTEMBER OF 1969, AND SEROLOGIC SURVEYS IN THE TWO SPECIES OF WILD RATS MENTIONED ABOVE. THE RATS WERE USED AS INDICATORS OF THE NATURAL OCCURRENCE OF TOXOPLASMA ON ISLANDS INHABITED BY CATS, COMPARED TO ISLANDS WHERE CATS HAD NEVER BEEN INTRODUCED. SEROEPIDEMIOLOGIC STUDIES WERE CONDUCTED ON MICRONESIANS INHABITING A SMALL ATOLL IN THE CAROLINE ISLANDS, AND ON NEW GUINEANS ATTENDING MISSION SCHOOLS WHERE THEY WERE IN CONTACT WITH CATS FOR THE FIRST TIME IN THEIR LIVES.

HUMAN DISEASE. FOUR CASES OF CONGENITAL TOXOPLASMOSIS, TWO SEVERE AND ONE FATAL, HAVE BEEN DIAGNOSED IN OUR LABORATORY IN THE PAST 8 MONTHS. THERE HAD BEEN ONLY ONE OTHER CASE ON RECORD IN HAWAII. THESE CASES, AND ANTIBODY PREVALENCE DATA FROM TESTS ON OAHU RESIDENTS, SUGGESTED THAT CONGENITAL TOXOPLASMOSIS MIGHT BE OCCURRING AT A RELATIVELY HIGH RATE IN HAWAII AND WOULD BE WORTHWHILE INVESTIGATING. OF PARTICULAR RESEARCH INTEREST, IS THE POSSIBILITY THAT A SIGNIFICANT NUMBER OF BABIES ARE BEING BORN WITH SUBCLINICAL INFECTIONS THAT REMAIN ACTIVE AND EVENTUALLY CAUSE SERIOUS CENTRAL NERVOUS SYSTEM DAMAGE. ADDITIONAL INFORMATION ON HUMAN CONGENITAL TOXOPLASMOSIS WAS OBTAINED THROUGH A PROSPECTIVE STUDY THAT INVOLVED SEROLOGIC SCREENING OF UMBILICAL CORD BLOOD COLLECTED FROM ALL BABIES BORN AT A MATERNITY HOSPITAL ON OAHU (ABOUT 50% OF THE BIRTHS IN HAWAII). BABIES WITH TOXOPLASMA ANTIBODY TITERS SUGGESTIVE OF ACTIVE INFECTION WERE FOLLOWED SEROLOGICALLY (INCLUDING ANTI-TOXOPLASMA IgM DETERMINATIONS) UNTIL TOXOPLASMOSIS WAS DIAGNOSED, OR RULED OUT. BABIES WITH SUBCLINICAL INFECTIONS WERE EVALUATED PERIODICALLY BY PSYCHO-PHYSIOLOGICAL TESTING PROCEDURES FOR EVIDENCE OF CENTRAL NERVOUS SYSTEM ABNORMALITIES.

MAJOR FINDINGS

CATS. OOCYSTS PROVEN TO BE TOXOPLASMA BY MOUSE INOCULATION WERE FOUND IN THE FECES OF 12 OF 1,604 STRAY OR UNWANTED CATS EXAMINED. ALTHOUGH THE NUMBER WAS SMALL, IT WAS THE FIRST TIME THAT TOXOPLASMA HAD BEEN ISOLATED FROM FECES OF CATS IN THEIR NATURAL HABITATS. MOST OF THE CATS IN THE STUDY

WERE SEROLOGICALLY TESTED (DYE TEST) AND THESE DATA WERE ANALYSED ACCORDING TO THE RELATIVE AGE OF THE CAT. OF A TOTAL OF 1,568 CATS TESTED FOR ANTIBODY, 14% WERE POSITIVE. ONLY ABOUT 5% OF CATS ESTIMATED TO BE LESS THAN 4 MONTHS OF AGE HAD ANTIBODY, WHEREAS 14% OF CATS 4-5 MONTHS OLD AND 23% OF CATS 6 MONTHS OF AGE OR OLDER HAD ANTIBODY.

EXPERIMENTALLY, SUSCEPTIBLE CATS USUALLY BECAME INFECTED AND PRODUCED OOCYSTS FOLLOWING THE CONSUMPTION OF CYSTS IN THE TISSUES OF CHRONICALLY INFECTED SMALL MAMMALS OR BIRDS; WHEREAS, CATS ADMINISTERED OOCYSTS BY THE ORAL ROUTE USUALLY DID NOT DEVELOP A PATENT INFECTION, OR ANTIBODY, UNLESS EXTRAORDINARILY LARGE DOSES WERE USED. THIS INFORMATION AND THE LACK OF ANTIBODY IN YOUNG CATS OBSERVED IN NATURE INDICATES THAT INTERMEDIATE HOSTS ARE NECESSARY IN THE LIFE CYCLE OF TOXOPLASMA.

INTERMEDIATE HOSTS. EXPERIMENTALLY, R. RATTUS AND R. EXULANS WERE EASILY INFECTED WITH OOCYSTS, ALTHOUGH ONE SPECIES WAS MORE RESISTANT THAN THE OTHER. THEY USUALLY DEVELOPED MEDIUM TO HIGH ANTIBODY TITERS AND HARBORED VIABLE CYSTS IN THE BRAIN AND OTHER TISSUES FOR LONG PERIODS OF TIME. OF SEVERAL HUNDRED RATS OF THESE SPECIES TRAPPED ON ISLANDS WHERE CATS WERE PRESENT, AN AVERAGE OF ABOUT 5% HAD TOXOPLASMA ANTIBODY, BUT 100 TO 200 RATS TRAPPED ON EACH OF FOUR SMALL ISLANDS WHERE CATS HAD NOT BEEN PRESENT, WERE ALL NEGATIVE.

A LIMITED SEROLOGIC SURVEY AND EXPERIMENTATION ON SEVERAL SPECIES OF WILD BIRDS IN HAWAII (REPORED PREVIOUSLY) SUGGESTED THAT ONLY COLUMBIDS (PIGEONS AND DOVES) WERE NATURALLY INFECTED. HOWEVER, RECENT EXPERIMENTS DEMONSTRATED THAT FRINGILLIDS (SPARROWS AND CARDINALS) DEVELOPED CHRONIC INFECTIONS WITH CYST FORMATION BUT NOT ANTIBODY DETECTABLE WITH THE DYE TEST.

LIZARDS IN THE GECKO FAMILY EXPOSED TO TOXOPLASMA OOCYSTS DID NOT BECOME INFECTED BUT IN SOME INSTANCES RETAINED OOCYSTS IN A VIABLE CONDITION IN THEIR DIGESTIVE TRACTS FOR SEVERAL DAYS.

IN SUMMARY, THE DATA OBTAINED INDICATE THAT CERTAIN SMALL WILD RODENTS AND BIRDS ARE GOOD INTERMEDIATE HOSTS OF TOXOPLASMA, BUT THEY ARE NOT FOUND NATURALLY INFECTED WITH THE PARASITE IN GEOGRAPHIC AREAS WHERE CATS DO NOT OCCUR. THIS IS FURTHER EVIDENCE THAT THE CAT IS ESSENTIAL IN THE LIFE CYCLE OF THE PARASITE. GECKOS DO NOT APPEAR TO BE INTERMEDIATE HOSTS BUT MAY SERVE AS TRANSPORT HOSTS (TO ANIMALS OTHER THAN CATS).

TRANSPORT HOSTS. EXPERIMENTS ON TWO SPEICES OF COCKROACHES, PERIPLANETA AMERICANA AND LEUCOPHAEA MADERAE, DEMONSTRATED THAT AFTER FEEDING ON CAT FECES CONTAINING OOCYSTS THESE INSECTS CAN HARBOR INFECTIOUS OOCYSTS IN THEIR DIGESTIVE TRACTS FOR AT LEAST THREE WEEKS, AND SHED THEM IN THEIR FECES FOR UP TO 10 DAYS. FURTHERMORE, COCKROACH FECES STORED AT ROOM TEMPERATURE AND HUMIDITY REMAINED INFECTIVE FOR MICE FOR ALMOST THREE WEEKS. CONSIDERING THEIR HABITS, COCKROACHES HAVE CONSIDERABLE POTENTIAL TO TRANSMIT TOXOPLASMA OOCYSTS TO HUMAN FOOD. ALSO THEY COULD SERVE AS A SOURCE OF INFECTION TO SMALL MAMMALS AND BIRDS THAT USE INSECTS AS FOOD.

SEROEPIDEMIOLOGIC STUDIES IN MAN. A DETAILED STUDY OF TOXOPLASMOSIS ON A SMALL ECOLOGICALLY-SIMPLE ATOLL IN THE WESTERN PACIFIC WAS RECENTLY COMPLETED. THE ONLY INHABITED ISLET (0.12 SQ. MI. IN AREA) IN THE ATOLL HAD A POPULATION OF ABOUT 300 PEOPLE, 34 CATS, 27 PIGS, 300 CHICKENS, NUMEROUS LAND AND SEA BIRDS AND NUMEROUS INSECTS INCLUDING FLIES AND COCKROACHES. BLOOD SPECIMENS COLLECTED FROM MOST OF THE HUMAN INHABITANTS WERE TESTED FOR TOXOPLASMA ANTIBODY AND 77% WERE POSITIVE. THERE WAS A LINEAR INCREASE IN ANTIBODY PREVALENCE WITH AGE, SUGGESTING AN ANNUAL SEROLOGIC CONVERSION OF 5 TO 10% UNTIL AGE 20 WHEN ABOUT 95% HAD ACQUIRED ANTIBODY. ANIMALS TESTED FOR ANTIBODIES INCLUDED 27 CATS, 27 PIGS AND 256 WILD RATS. EIGHTY SIX PERCENT OF THE CATS, 25% OF THE PIGS AND 4% OF THE RATS WERE POSITIVE. CAREFUL OBSERVATIONS ON THE HABITS OF THE PEOPLE, AND SPECIFIC INFORMATION COLLECTED ON DIET, RULED OUT MEAT AS A MAJOR SOURCE OF HUMAN INFECTION. CONTAMINATION OF HUMAN FOOD WITH TOXOPLASMA OOCYSTS BY TRANSPORT HOSTS SUCH AS FLIES OR COCKROACHES APPEARED TO BE A LIKELY METHOD OF HUMAN INFECTION. IF MOST INFECTIONS HAD BEEN THE RESULT OF DIRECT CONTACT WITH OOCYSTS ON CATS OR IN THE SOIL, A RELATIVELY HIGHER PREVALENCE OF ANTIBODY IN THE YOUNGER AGE GROUP SHOULD HAVE BEEN OBSERVED.

AS REPORTED PREVIOUSLY, SERA COLLECTED BY DR. GAJDUSEK FROM NATIVES LIVING IN REMOTE AREAS OF NEW GUINEA WERE MOSTLY NEGATIVE FOR TOXOPLASMA ANTIBODY. THESE PEOPLE WERE OF INTEREST BECAUSE THEY INHABITED AREAS OF NEW GUINEA WHERE FELIDS HAD NEVER BEEN PRESENT. THIS YEAR, TO CONTROL THESE STUDIES, DR. GAJDUSEK PROVIDED SERA FROM PEOPLE BELONGING TO THE SAME TRIBES BUT ATTENDING MISSION SCHOOLS IN OTHER AREAS WHERE CATS WERE PRESENT. ANTIBODY TO TOXOPLASMA WAS FOUND IN A NUMBER OF THESE PEOPLE. SERA WERE ALSO OBTAINED FROM NATIVES LIVING ON A SMALL ISLAND ABOUT 200 MILES FROM NEW GUINEA WHERE THERE HAD BEEN NO CATS, AT LEAST IN RECENT TIMES. OF 70 PEOPLE 9 TO 34 YEARS OF AGE, ANTIBODY WAS PRESENT IN ONLY 2, COMPARED TO THE PRESENCE OF ANTIBODY IN 28 OF 70 PEOPLE 35 TO 66 YEARS OF AGE.

HUMAN DISEASE. THE PROSPECTIVE STUDY ON CONGENITAL TOXOPLASMOSIS IN HAWAII WAS INITIATED EARLY IN FEBRUARY, 1972. OF 639 UMBILICAL CORD SERA TESTED TO DATE, HIGH DYE-TEST TITERS ($\geq$ 512) WERE FOUND IN 25. OF THESE, EIGHT PROBABLY HAD MATERNAL ANTIBODY, ONE PROBABLY WAS INFECTED CONGENITALLY AND THE OTHERS WILL REQUIRE FURTHER SEROLOGIC TESTS BEFORE DETERMINATIONS CAN BE MADE. THE INFECTED BABY APPEARED TO BE NORMAL.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE

HUMAN TOXOPLASMOSIS IS RECOGNIZED AS A MEDICAL PROBLEM THROUGHOUT THE WORLD BUT THE PRINCIPAL METHOD (OR METHODS) BY WHICH MAN BECOMES INFECTED IS NOT YET CLEAR. MORE DEFINITIVE INFORMATION ON TRANSMISSION WOULD BE DESIRABLE. THE PREVALENCE OF HUMAN INFECTION WITH TOXOPLASMA IS USUALLY HIGH IN UNDEVELOPED AREAS OF THE TROPICS AND SUBTROPICS, BUT MORE INFORMATION ON THE RELATIVE PUBLIC HEALTH IMPORTANCE OF TOXOPLASMOSIS IN SUCH AREAS IN NEEDED. HAWAII AND OTHER PACIFIC ISLANDS OFFER UNIQUE OPPORTUNITIES TO GAIN INSIGHT INTO THE LIFE HISTORY AND TRANSMISSION OF TOXOPLASMA GONDII, AND TO EVALUATE THE PUBLIC HEALTH IMPORTANCE OF THIS PARASITE.

PROPOSED COURSE OF THE PROJECT

AT THIS TIME, FURTHER EXPERIMENTATION AND FIELD STUDIES ON CATS AND SUSPECTED INTERMEDIATE AND TRANSPORT HOSTS DO NOT APPEAR TO BE WARRANTED. UNIQUE EPIDEMIOLOGIC SITUATIONS, HOWEVER, WILL BE INVESTIGATED WHEN FEASIBLE. OF PARTICULAR INTEREST ARE HUMAN POPULATIONS LIVING ON ISLANDS WHERE CATS HAVE NOT BEEN INTRODUCED.

INVESTIGATION OF THE PUBLIC HEALTH IMPORTANCE OF TOXOPLASMOSIS IN THE PACIFIC AREA WILL BE EMPHASIZED AND SHOULD PRODUCE INFORMATION APPLICABLE TO OTHER TROPICAL AND SUBTROPICAL AREAS AS WELL AS THE PACIFIC. THE STUDY ON CONGENITAL TOXOPLASMOSIS, IF SUFFICIENT CASES ARE FOUND, PROVIDES THE POTENTIAL TO EVALUATE THE LONG TERM RESIDUAL EFFECTS OF INAPPARENT INFECTION, A SUBJECT ON WHICH LITTLE IS KNOWN. OF COURSE, IF ONLY A FEW CASES OF CONGENITAL INFECTION ARE FOUND, THE STUDY WILL BE TERMINATED.

IN SOME PACIFIC ISLAND POPULATIONS OUTSIDE OF HAWAII A HIGH PREVALENCE OF TOXOPLASMA ANTIBODY INDICATES THAT CONGENITAL DISEASE IS NOT A PROBLEM BECAUSE MOST OF THE FEMALES HAVE BEEN INFECTED, AND ARE IMMUNE, WHEN REACHING CHILD BEARING AGE. AN IMPORTANT QUESTION, HOWEVER, IS WHETHER ACQUIRED TOXOPLASMOSIS IS CAUSING A SIGNIFICANT NUMBER OF CASES OF CHORIORETINITIS IN SUCH POPULATIONS. SINCE INFORMATION ON THIS QUESTION IS OF IMPORTANCE, INVESTIGATIONS IN COLLABORATION WITH INTERESTED OPHTHALMOLOGISTS ARE BEING PLANNED.

HONORS AND AWARDS:

DR. WALLACE CONTINUED TO SERVE AS ASSISTANCE CLINICAL PROFESSOR OF PUBLIC HEALTH AND ASSISTANT CLINICAL PROFESSOR OF TROPICAL MEDICINE AND MEDICAL MICROBIOLOGY IN THE UNIVERSITY OF HAWAII SCHOOLS OF PUBLIC HEALTH AND MEDICINE, RESPECTIVELY.

PUBLICATIONS:

WALLACE, G.D.: EXPERIMENTAL TRANSMISSION OF TOXOPLASMA GONDII BY FILTH-FLIES. AMER. J. TROP. MED. HYG., 20:411-413, 1971.

WALLACE, G.D.: ISOLATION OF TOXOPLASMA GONDII FROM THE FECES OF NATURALLY INFECTED CATS. J. INFECT. DIS., 124:227-228, 1971.

WALLACE, G.D., MARSHALL, L., AND MARSHALL, M.: CATS, RATS, AND TOXOPLASMOSIS ON A SMALL PACIFIC ISLAND. AMER. J. EPIDEM., 1972 (IN PRESS)

Serial No. NIAID 67F

1. Laboratory of Parasitic Diseases
2. Pacific Research Section
3. Honolulu, Hawaii

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Studies on competition between Aedes albopictus and Aedes polynesiensis in nature.

Previous Serial Number: Same

Principal Investigator: Dr. Leon Rosen

Other Investigators: Dr. Lloyd Rozeboom, Dr. Duane Gubler, Dr. Jean Lagraulet.

Cooperating Units: School of Hygiene and Public Health, Johns Hopkins University, Baltimore; Institut de Recherches Medicales, Papeete, Tahiti.

Man Years:	NIAID staff	Guest workers
Total:	1/12	1/12
Professional:	1/12	1/12
Others:	0/12	

Project Description:

Objectives

Aedes polynesiensis is a member of the scutellaris group of mosquitoes and the major vector of human filariasis (Wuchereria bancrofti) in Polynesia and parts of Melanesia. It is impractical to control this species by conventional means because of the great diversity of its small breeding sites and the fact that the adults do not rest in houses. Aedes albopictus is a related species of mosquito which does not occur naturally in the same geographic area as A. polynesiensis. It is completely refractory to infection with human filariae. Observations on A. albopictus in areas where it coexists with other members of the scutellaris group suggest that if A. albopictus were deliberately introduced into an area where a scutellaris species occurred, the latter might be greatly reduced in numbers, or even eliminated. Competition between both larval and adult forms of A. albopictus and A. polynesiensis has been studied intensively in the laboratory and these data also suggest that A. albopictus might greatly reduce the population of A. polynesiensis if the

former were introduced into an area where the latter occurred. The objective of this project is to study the effect of such an introduction on an isolated island which harbors a natural population of A. polynesiensis.

Methods Employed

A heavily wooded coral atoll with an abundant A. polynesiensis population was selected as the locale for the experimental introduction of A. albopictus. baseline population estimates of A. polynesiensis were made three times at each of a number of selected sites on the island at three-month intervals prior to the introduction of A. albopictus. Three strains of A. albopictus were released at widely separated localities on the island in January-February, 1970. One strain consisted of adults reared directly from eggs deposited by wild females on Oahu, Hawaii. The second consisted of adults reared from eggs from a laboratory colony originally started with specimens from Oahu, and the third consisted of adults reared from eggs from a laboratory colony originally started with specimens from Poona, India. It was estimated that between 3000 and 6000 females (and a comparable number of males) of each strain were released. Many of the females were given a blood meal in the laboratory prior to their release. Follow-up visits were made to the atoll in May and August, 1970, February, 1971, and January, 1972 to assess the fate of the liberated species.

Major Findings

At the end of the first year following release, one of the three strains liberated (Poona) appeared to have died out and A. albopictus larvae and adults were less abundant than they were at the three- and six-month follow-up surveys. At that time this species was not more abundant in the vicinity of artificial larvae breeding sites nor did it favor the small part of the island inhabited by people and domestic animals. At the end of the second year following release, A. albopictus larvae and adults were still present on the island but only in very small numbers (7 of 2412 larvae and 3 of 1380 adults examined). In retrospect, it appeared that this species had been declining steadily since its introduction and that succeeding generations had been unable to replace themselves.

Although it is not certain that A. albopictus will disappear from the atoll, it appears likely that it will. It is not known if the apparent inability of A. albopictus to become established is the result of 1) the relatively small numbers of specimens released, 2) an inability of this species to become adapted to the relatively harsh conditions of atoll environment, or 3) competition from A. polynesiensis. A combination of the first and second factors seems the most likely explanation of the results to date.

Significance to Biomedical Research and the Program of the Institute

If the present experimental approach is ultimately successful, it will provide a means of controlling, or even eradicating, human filariasis in the entire endemic area in Polynesia and parts of Melanesia at little or no cost. The experiment also has significance in that it provides an opportunity to make observations on the population dynamics of a newly introduced mosquito species under controlled conditions. In view of the current interest in the control of vector species by biologic (rather than chemical) methods, the data obtained from this project should be of considerable value.

Proposed Course of the Project

Despite the discouraging results from Taiaro atoll it is judged worthwhile to make at least one more attempt to determine if A. albopictus can displace A. polynesiensis in nature - as it does in the laboratory. It is tentatively planned to carry out the second introduction on a volcanic island (Rarotonga, Cook Islands or possibly American Samoa) and to release far larger numbers of A. albopictus over a longer period of time than was done on Taiaro.

Honors and Awards:

See NIAID 67C

Publications:

Rosen, L.: Angiostrongyliasis. In Hubbert, W.T., McCulloch, W.F., and Schnurrenberger, P.R. (eds.): Diseases Transmitted from Animals to Man - 6th Edition, (in press).

SERIAL NO. NIAID 67H

1. LABORATORY OF PARASITIC DISEASES
2. PACIFIC RESEARCH SECTION
3. HONOLULU, HAWAII

PHS-NIH
INDIVIDUAL PROJECT REPORT
JULY 1, 1971 THROUGH JUNE 30, 1972

PROJECT TITLE: STUDIES ON HUMAN INFECTION WITH ISOSPORA FELIS, A COCCIDIUM OF CATS.

PREVIOUS SERIAL NUMBER: NONE

PRINCIPAL INVESTIGATOR: DR. GORDON D. WALLACE

OTHER INVESTIGATORS: DR. TIMOTHY T. KUBERSKI

COOPERATING UNITS: NONE

MAN YEARS:

	NIAID STAFF
TOTAL:	4/12
PROFESSIONAL:	4/12
OTHER:	0/12

PROJECT DESCRIPTION:

OBJECTIVES

TO DETERMINE IF MAN IS NATURALLY INFECTED WITH ISOSPORA FELIS.

METHODS EMPLOYED

A SEROLOGIC TEST FOR I. FELIS HAS BEEN DEVELOPED UTILIZING THE INDIRECT FLUORESCENT ANTIBODY TECHNIQUE. BOTH THE SPOROZOITE AND MEROZOITE STAGE OF THE PARASITE WERE USED AS ANTIGEN. SPOROZOITES WERE OBTAINED BY EXCYSTING SPOROCYSTS BY ULTRASOUND FROM OOCYSTS AND MONKEY KIDNEY CELL CULTURES INOCULATED WITH SPOROZOITES WERE THE SOURCE OF MEROZOITES. BECAUSE OF DIFFICULTIES IN OBTAINING SUFFICIENT NUMBERS OF MEROZOITES, SPOROZOITES WERE USED AS ANTIGEN IN MOST INSTANCES.

MAJOR FINDINGS

WHAT APPEARS TO BE SPECIFIC ANTIBODY TO I. FELIS HAS BEEN FOUND IN SERUM OF MICE INOCULATED ORALLY WITH I. FELIS OOCYSTS. SERA COLLECTED FROM MICE BEFORE THEY WERE EXPOSED TO OOCYSTS DID NOT REACT WITH THE SPOROZOITES (NO FLUORESCENCE), COMPARED TO SERA COLLECTED FROM THE SAME MICE THREE WEEKS AFTER THEY WERE EXPOSED TO OOCYSTS THAT PRODUCED A STRONG FLUORESCENT STAINING

OF THE PARASITES. THE TITER OF SERUM FROM ONE MOUSE WAS GREATER THAN 1:512. SERA FROM MICE THAT REACTED WITH I. FELIS ANTIGEN DID NOT REACT WITH TOXOPLASMA ANTIGEN AND MOUSE SERA WITH TOXOPLASMA ANTIBODY DID NOT REACT WITH I. FELIS ANTIGEN.

SIGNIFICANCE TO BIOMEDICAL RESEARCH AND THE PROGRAM OF THE INSTITUTE

IF TOXOPLASMA INFECTION IN MAN IS USUALLY ACQUIRED FROM EXPOSURE TO THE OOCYST STAGE OF THIS PARASITE (EXCRETED IN CAT FECES) AS IS NOW GENERALLY PRESUMED, THEN MAN SIMILARLY WOULD BE EXPOSED TO OTHER COCCIDIA OF CATS. DR. JACK FRENKEL AND ASSOCIATES HAVE ALREADY DEMONSTRATED THAT STAGES OF THE CAT COCCIDIA ISOSPORA FELIS AND ISOSPORA RIVOLTA INVADE EXTRA-INTESTINAL TISSUES OF THE HOST. ALSO THEY HAVE SHOWN THAT MICE AND OTHER RODENTS CAN BE INFECTED AND SERVE AS INTERMEDIATE HOSTS OF THE PARASITE, SHORTENING THE PREPATENT PERIOD IN THE HOST. SINCE IT APPEARS THAT PATENT I. FELIS INFECTIONS ARE MORE COMMON IN THE CAT THAN PATENT TOXOPLASMA INFECTIONS, HUMAN EXPOSURE TO I. FELIS IS PROBABLY GREATER THAN TO TOXOPLASMA. IT WOULD BE IMPORTANT TO LEARN IF THIS PARASITE DOES INFECT MAN.

PROPOSED COURSE OF THE PROJECT

IN THE NEAR FUTURE SELECTED HUMAN SERA WILL BE TESTED FOR I. FELIS ANTIBODY. IF ANTIBODY IS NOT FOUND IN HUMAN SERA, OR IS PRESENT ONLY AT A VERY LOW PREVALENCE, THE PROJECT WILL BE TERMINATED. IF ANTIBODY IS FOUND IN A SIGNIFICANT PROPORTION OF SERA, STUDIES ON ITS SPECIFICITY, ON THE OCCURRENCE OF DISEASE, AND ON THE EPIDEMIOLOGY OF INFECTION WOULD BE INDICATED. THE POSSIBILITY OF HUMAN INFECTION WITH OTHER COCCIDIA OF CATS AND COCCIDIA OF OTHER ANIMALS WOULD ALSO NEED TO BE INVESTIGATED.

HONORS AND AWARDS: SEE NIAID PROJECT 67D

PUBLICATIONS: NONE

LABORATORY OF IMMUNOLOGY

Summary 1

Serial No.

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PHS-NIH
Summary Statement
Office of the Chief
Laboratory of Immunology
July 1, 1971 through June 30, 1972

This past year has been one of considerable productivity and scientific progress for the Laboratory of Immunology. Dr. Myron Waxdal joined the Laboratory in June of 1972. A highly equipped and automated protein chemistry laboratory has been created to fill a major need within the laboratory, and Dr. Waxdal and Dr. Inman will staff this important facility. Four associates, Dr. John Breitner, Dr. Benjamin Cohen, Dr. Gerald Elfenbein and Dr. Michael Harrison entered the laboratory this year. In addition, Dr. Mariella Carta-Sorcini was appointed as a visiting fellow and Dr. Takeshi Yoshida served as a visiting scientist.

Four associates, Drs. Joseph Davie, Leonard Ellman, David Katz and John Winfield completed their assignments at the beginning of this fiscal year, although Dr. Davie has maintained a relationship with the Laboratory, while serving as a resident in the Laboratory of Pathology, NCI. Each of these associates has had a substantial record of achievement during his tenure in the Laboratory.

The members of the Laboratory have played an increasingly important role within the immunologic community. Several new appointments to editorial boards of immunology journals were made this year. Dr. Paul was appointed to the editorial boards of the Journal of Immunology, Immunochemistry, the Scandinavian Journal of Immunology and Immunological Communications and to the board of reviewers of Federation Proceedings; Dr. Mage now serves on the editorial boards of Immunochemistry and Federation Proceedings; Dr. Green and Dr. Inman are members of the board of reviewers of Federation Proceedings and Dr. Merchant was appointed to the editorial board of Infection and Immunity. Miss Lieberman, Dr. Green and Dr. Merchant chaired sessions at the annual meeting of the American Association of Immunologists and Dr. Mage and Dr. Paul served as members of the Program Committee for that meeting. Dr. Mage has recently joined the Allergy and Immunology Study Section.

In addition, Dr. Paul chaired sessions at the Conference on the Immunology of Carcinogenesis at Gatlinburg, Tennessee and at the Fogarty Center Conference on the Biology of Mammalian Cell Membranes. Dr. Green also chaired a session at that Conference. Dr. Green, Miss Lieberman and Dr. Merchant were invited participants at the 5th Brook Lodge Symposium on Genetic Control of Immune Responses and Disease Susceptibility and Dr. Paul was a major speaker at the Third International Convocation on Immunology. In addition, members of the Laboratory have given seminars at many major universities in the United States and Canada. Dr. Bruce Merchant now serves as the Equal Employment Opportunity Counselor for NIAID.

The Laboratory of Immunology has as its basic goal the exploration of fundamental mechanisms in cellular immunology, immunogenetics, immunochemistry, immunopathology and tumor immunology. To these ends, studies of the function

and nature of antigen-binding receptors, of the extent of lymphocyte heterogeneity and the mechanisms of lymphocyte interactions, of the process of tolerance induction and of the nature, genetics and mode of function of specific immune response genes are being actively pursued. Mechanisms of cell mediated cytotoxicity, of the nature of the genetic control of immunoglobulin synthesis and variability and of the control of activation of immunocompetent cells are also being intensively studied.

HETEROGENEITY OF THYMUS-DERIVED LYMPHOCYTES

Two major classes of lymphocytes, the thymus-derived (T) and bone-marrow derived (B), have been previously described and shown to subserve independent functions. The T lymphocytes are principally involved in the various forms of cellular immune responses. Dr. John Stobo and Dr. William Paul of the Laboratory of Immunology have now demonstrated the existence of at least two subclasses of T cells. These cells differ from each other in the following respects. One type of cell is principally in the recirculating pool, is abundant within the lymph node lymphocyte population, is sensitive to irradiation and to anti-lymphocyte serum, and is relatively rich in the surface differentiation antigen θ. This cell responds to the mitogens phytohemagglutinin and concanavalin A. The other major class of T cells is relatively sessile, is found primarily in the spleen, is relatively resistant to radiation and anti-lymphocyte serum and has a low density of surface θ. This second cell type responds well to concanavalin A but poorly to phytohemagglutinin. Recent studies indicate that these sets of T cells have different immunologic functions. Studies are now in progress to determine if these represent two independent T cell lines or differentiated cellular forms along a single T cell line. (Dr. J.D. Stobo, Dr. W.E. Paul)

ANTIGEN BINDING RECEPTORS OF LYMPHOCYTES

Lymphocytes which function as the precursors of antibody producing cells have been postulated to be stimulated as a result of the interaction of antigen with cell surface receptors. Dr. Joseph Davie and Dr. William Paul have demonstrated the existence of such receptor molecules on the surface of guinea pig lymphocytes. A model antigen, the dinitrophenyl derivative of guinea pig albumin (DNP-GPA), is specifically bound by approximately 40 lymphocytes per 100,000 lymph node cells. These antigen-binding cells increase markedly in number after immunization and are dramatically diminished in number in tolerant animals. Moreover, methods have been developed to study the number of receptors per antigen-binding cell and the binding affinity of such receptors. These studies demonstrate that each antigen-binding cell has approximately 60,000 receptors and the average affinity of receptors increases as time after immunization increases. Studies are now in progress to evaluate changes in receptors after these receptors have bound antigen. The ability to precisely study the binding of antigen to receptors and the immediate cellular consequences of this interaction represent an important step in the delineation of the mechanism by which antigens activate immunocompetent cells. (Dr. J.M. Davie, Dr. W.E. Paul)

DEMONSTRATION OF TWO INDEPENDENT IMMUNE RESPONSE GENE LOCI

Immune response (IR) genes control the capacity of various animals to mount specific immune responses to certain groups of antigen. Rose Lieberman and William Humphrey of this Laboratory have recently described two new IR genes in mice, one controlling the immune response to IgA myeloma proteins and the other, the response to IgG myeloma proteins. These genes are termed IR-IgA and IR-IgG respectively. Both of these genes are linked to the major histocompatibility system (H2) of mice. Recently, Lieberman and her colleagues have obtained definitive evidence that at least two independent IR loci exist one for Ir-IgG and one for Ir-IgA and, therefore, that these IR genes are not allelic. In collaboration with Dr. J. Stimpfling (Columbus Hospital), an examination of mice whose H2 region were derived by genetic crossover events, yielded one strain in which the crossover had occurred between the Ir-IgA and Ir-IgG, thus demonstrating the independence of these loci. Moreover, since the distinction between the genes was accomplished by testing only 5 recombinant strains, it is likely that Ir-IgA and Ir-IgG are relatively far apart and that many Ir genes exist. As Ir genes may be important in disease susceptibility, precise information concerning their genetics and function promises to have wide biologic and potential clinical importance. (R. Lieberman, W. Humphrey, Dr. J. Stimpfling, Columbus Hospital)

INHIBITION OF IMMUNE RESPONSE GENE FUNCTION BY ALLOANTISERA

The mechanism by which specific histocompatibility-linked immune response (IR) genes mediate their function and the nature of their gene product are unknown. Studies performed by Drs. Ethan Shevach, William Paul and Ira Green in the Laboratory of Immunology have demonstrated that the products of specific guinea pig IR genes function at the cell surface. The immune response to the 2,4-dinitrophenyl (DNP) copolymer of L-glutamic acid and L-lysine (GL) is controlled by an IR gene linked to the strain 2 histocompatibility gene complex; the immune response to the copolymer of L-glutamic acid and L-tyrosine (GT) is controlled by an IR gene linked to the strain 13 histocompatibility gene complex. (2 x 13)F1 guinea pigs possess both IR genes and thus respond to both DNP-GL and GT. If peritoneal exudate lymphocytes from F_1 guinea pigs immunized to DNP-GL and GT emulsified in complete Freund's adjuvant are cultured in normal guinea pig serum, there is an *in vitro* cellular response (stimulation of DNA synthesis) to both DNP-GL, and GT and to the mitogenic agent phytohemagglutinin (PHA). If the cells are cultured in an allo-antisera produced by immunizing strain 2 guinea pigs with strain 13 lymphoid cells, there is a normal response to DNP-GL and PHA but no response to GT. If an anti-2 antisera is used, the response to GT and PHA is normal but the response to DNP-GL markedly suppressed. A mixture of both sera suppresses the response to both GT and DNP-GL but not to PHA. This experiment provides strong evidence for the existence of IR gene products on the surface of thymus derived lymphocytes and offers a powerful model to determine the chemical nature and mechanism of action of IR gene products. (Dr. E.M. Shevach, Dr. W.E. Paul, Dr. I. Green)

SPECIFICITY OF ACTIVATION OF IMMUNOCOMPETENT CELLS

In previous studies from the Laboratory of Immunology, Drs. Merchant and Inman have demonstrated that the activation of precursors of antibody secreting cells may be less specific than had previously been believed. They

demonstrated that in response to immunization with hapten-derivatized erythrocytes many antibody producing cells appeared which lysed erythrocytes coated with alternate, but related, haptens more effectively than those coated with the immunizing hapten. The number of these cells was often markedly in excess of those producing highly specific antibody and this effect was more prominent in the population of cellsproducing IgM antibody than in the IgG producers. They have now demonstrated that inbred mice demonstrate very precise and reproducible numbers of cells producing antibody to each of a large series of alternate hapten-erythrocyte conjugates whereas non-inbred animals demonstrate much greater variability. This suggests that genetic factors have a very prominent role in the specific activation of precursor cells. (Dr. E.B. Merchant, Dr. J.K. Inman)

DEMONSTRATION OF INDEPENDENT VARIABLE AND CONSTANT REGION GENES FOR IMMUNOGLOBULIN HEAVY CHAINS

Immunoglobulins consist of two major types of polypeptide chains--heavy (H) and light (L). In turn,these chains consist of regions of high variability (V regions) and regions of high constancy (C regions). Genetic studies from the Laboratory of Immunology have strongly suggested that independent, but closely linked, genes control the V region and the C region, respectively, of rabbit H chains. This analysis was made possible by the existence of stable genetic markers (allotypes) associated with V regions and C regions. The V region marker is controlled by the a locus and the C region marker of γG immunoglobulin by the A14 or A15 alleles. In the examination of a large number of progeny of matings of animals typed for these markers, a single animal was noted, the immunoglobulin of which had allelic markers indicative of a genetic crossover between the V region and C region genes. Dr. Mage in association with Dr. Carta, Dr. Appella (NCI) and Dr. Reynolds (Laboratory of Clinical Investigation) have demonstrated that this "recombinant" animal transmitted to its offspring immunoglobulin genes of the recombinant type. Moreover, these "recombinant" immunoglobulins were of normal size and had the tryptic peptides expected. These new studies strongly reinforce the previous evidence for independent V and C region genes and aid considerably in our understanding of the genetic control of antibody synthesis and diversity. (Dr. R. Mage, Dr. M. Carta, Dr. E. Appella, NCI, Dr. H. Reynolds, LCI)

ANTIBODY DEPENDENT LYMPHOCYTE MEDIATED CYTOTOXICITY: MEDIATION BY BONE MARROW DERIVED LYMPHOCYTES

Lymphoid cells from non-immune animals are capable of causing the lysis, in vitro, of target cells coated with specific antibody. Studies from this Laboratory have previously demonstrated that thymus-derived (T) lymphocytes, which are the effector cells in most forms of cellular hypersensitivity, are not responsible for this effect. Van Boxel,Green and Paul, of the Laboratory of Immunology, together with Dr. Michael Frank of the Laboratory of Clinical Investigation, have obtained direct evidence that this antibody dependent target cell lysis is mediated by a bone marrow-derived (B) lymphocyte. The experiments demonstrating this have involved the specific removal of B lymphocytes from a mouse spleen cell population by the formation of rosettes with sheep erythrocytes coated with antibody and complement and subsequent density gradient

centrifugation. Only B lymphocytes, which possess a receptor for complement, are removed in this way. The spleen cell populations which have been depleted of B cells have a marked reduction in their ability to lyse antibody-coated target cells. Moreover, recovery and dissociation of the B cell rosettes yields a cell population with heightened activity. The capacity of B cells to mediate this response may reside in a B cell subpopulation as lymph node cells, despite the presence of some B cells, are quite inactive. The cooperative effect of B cells and antibody in target cell lysis may have important immunopathologic implications. (Dr. J.A. van Boxel, Dr. I. Green, Dr. W.E. Paul, Dr. Michael Frank, LCI)

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Receptors on Immunocompetent Cells

Previous Serial No.: Same

Principal Investigator: Dr. William E. Paul

Other Investigators: Dr. J.M. Davie, Lab. of Pathology, NCI
Dr. B. Cohen, LI, NIAID
Dr. J. C.S. Breitner, LI, NIAID
Dr. R. Sharon, LI, NIAID
Dr. A.S. Rosenthal, LCI, NIAID
Dr. R. Asofsky, LMI, NIAID

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	3-6/12	6/12		4
Professional	2-6/12	6/12		3
Other	1			1

Project Description:

Objectives:

The initiation of immune responses involves the specific interaction of antigen and cell surface receptor molecules. In this project, a study of the nature, distribution and affinity of these surface molecules on both bone marrow derived (B) and thymus derived (T) lymphocytes has been undertaken. Furthermore, the mechanism by which antigen-binding causes cell activation or, alternatively, inactivation (tolerance) is unknown and information on changes in antigen-binding lymphocytes after the contact of antigen with the cell offers a promising approach to the understanding of the cell biology of the response to antigen.

Methods Employed:

Lymphocyte receptors were evaluated by the capacity of cells to bind radiolabeled antigen and/or fluorescent anti-immunoglobulin to their surfaces.

The results were determined by preparation of autoradiographs and inspection under visible and ultraviolet illumination. In addition, measurement of total radioactivity associated with cell pellets by γ counting has been used. The induction of tolerance has been monitored by measurement of antibodies to the 2,4-dinitrophenyl (DNP) determinant following immunization with DNP-ovalbumin (DNP-OVA).

Major Findings:

Normal guinea pigs possess rare lymphocytes (~ 40 per 100,000) which specifically bind ^{125}I-DNP-guinea pig albumin (^{125}I-DNP-GPA) to their surface. These antigen-binding cells, which appear to be precursors of anti-DNP antibody secreting cells, have been demonstrated by combined immunofluorescence and autoradiography to possess large amounts of surface immunoglobulin. Moreover, the immunoglobulin appears to be the antigen-binding receptor as excess anti-immunoglobulin blocks the binding of antigen. In adult guinea pigs, the majority (60-70%) of the lymphocytes which bind ^{125}I-DNP-GPA possess surface immunoglobulin of the γ_2 heavy (H) chain class while approximately 25% of the antigen-binding lymphocytes possess immunoglobulin of the μ H chain class and a small proportion, γ_1 H chains. The situation differs during fetal life, however. DNP-GPA binding lymphocytes are detectable as early as 50 days of gestation in a frequency of ~ 20 per 100,000 lymphocytes. At this time, virtually all of these cells bear surface immunoglobulin of the μ class. Substantial numbers of γ_2 DNP-GPA binding cells are first noted at about the time of birth. Similarly, among immunoglobulin bearing cells, μ is the predominant class in fetal life, and γ_2 and γ_1 bearing cells gradually increase in number and proportion so that γ_2 bearing cells are most numerous in adults.

After immunization of guinea pigs with DNP-GPA, the number of DNP-GPA binding lymphocytes increases in number markedly. If the antigen is administered as an emulsion in complete Freund's adjuvant, the majority of the antigen binding cells have surface γ_2 immunoglobulin and γ_2 is the predominant type of antibody produced. On the other hand, immunization with DNP-GPA emulsified in incomplete Freund's adjuvant causes a selective increase in antigen-binding cells bearing surface γ_1 or μ immunoglobulin, with very little change in the number of γ_2 bearing cells. Again, this mode of immunization leads to the production of relatively little serum anti-DNP antibody of the γ_2 class.

Thus, the most primitive antigen-binding cells bear surface μ determinants. An apparently antigen-independent "switch" occurs either in individual cells or in the population leading to a predominance of γ_2 bearing cells. Immunization protocols favoring production of γ_2 antibody lead to an increase in γ_2 bearing cells whereas immunization protocols favoring the production of γ_1 antibody cause a marked increase in antigen-binding cells of the γ_1 class.

Not only does immunization cause an increase in the numbers of specific antigen-binding cells but the affinity with which the surface receptors bind

the antigen increases as well. Thus, the concentration of ε-DNP-L-lysine required to inhibit DNP-GPA binding lymphocytes from binding DNP-GPA falls rapidly during the first 30 days after immunization. 50% of the antigen-binding cells from non-immune guinea pigs are inhibited from binding ^{125}I-DNP-GPA by ~ 10^{-3} M ε-DNP-L-lysine while ~ 10^{-9} M suffices for cells from animals immunized 30 days earlier. This increase in the affinity of cell surface receptors of the population of antigen-binding cells is paralleled by an increase in the affinity of antibody secreted by individual antibody-producing cells and by an increase in equilibrium constant for the binding of ε-DNP-L-lysine by serum anti-DNP antibody. In order to analyze the cellular dynamics of this affinity increase, a simple technique for determining the number of antibody secreting (plaque-forming) cells producing antibody in one of several affinity ranges was developed. Using this procedure, it was demonstrated that high affinity antibody producing cells may be detected shortly after immunization and that maximum numbers of these cells occur quite early in the course of the immune response. The principal event leading to the increase in overall affinity of the population of antibody secreting cells is the rapid fall in the number of cells producing low affinity antibody. Through the use of in vivo administration of tritiated thymidine and subsequent in vitro enumeration of relative fractions of labeled and unlabeled cells producing antibody, it was shown that precursors of producers of high affinity antibody continue to proliferate after precursors of producers of low affinity antibody have ceased to divide.

This data strongly suggests that the phenomenon of increasing affinity of antibody as the interval after immunization increases is due to a selection by antigen among pre-existing precursors of antibody secreting cells. Furthermore, the data implies that little or no somatic mutation is involved in this process and that the principal mechanism of achieving the affinity increase is the specific failure of low affinity precursors to be stimulated as the concentration of available antigen falls and the amount of antibody rises.

Further evidence for increase in affinity of surface receptors on antigen-binding cells was gained from direct measurement of binding equilibrium and association and dissociation rates. The binding equilibrium was measured by determining the concentration of ^{125}I-DNP-GPA required to bind to half of all the antigen-binding receptors on precursor cells. Equilibrium constants calculated in this way increased with time after immunization and were in the range of 10^8 to 10^9 L/M. When association rates and dissociation rates were determined, it was noted that the association rate increased and the dissociation rate diminished with time also leading to an increasing equilibrium constant. However, the equilibrium constant calculated from association-dissociation data was considerably greater than that obtained by equilibrium methods. This discrepancy appeared principally due to an inordinately slow dissociation rate from which it was concluded that secondary slow reactions on the membrane stabilized the antigen-receptor complex. This study also yielded an estimate of the number of antigen binding receptors per specific cell (~ 60,000) and suggested that multivalent anti-

gens of relatively restricted size were initially bound by only 2 bonds to the cell surface.

In view of the possibility of secondary "slow" reactions on the cell surface and the reports which have appeared in the literature of migration of surface immunoglobulin over the membrane after the addition of anti-immunoglobulin, attempts to study the dynamics of bound antigen on the cell surface have been made. The procedures utilized thus far have involved the binding of radioactive or non-radioactive antigen to lymph node cells from immunized mice. These cells are then incubated for various periods of time, in the presence or absence of inhibitors. The surface bound antigen is then evaluated for 1) spontaneous release into the culture medium; 2) sensitivity to removal by trypsin and 3) availability to be bound by ^{125}I-anti-DNP. At this time, changes in all parameters occur but the precise significance of these changes is not yet certain. Concurrently, changes in membrane associated receptors are also being evaluated by electron-microscopic studies through the use of DNP-GPA-horseradish peroxidase and anti-immunoglobulin-horseradish peroxidase complexes.

In parallel to these studies, a series of investigations on antigen-binding and antibody secreting cells in partially or completely tolerant animals has been initiated. A state of DNP specific tolerance in guinea pigs and mice can be initiated through the use of a DNP conjugate of the non-immunogenic copolymer of D-glutamic acid and D-lysine (DNP-D-GL). The magnitude of the tolerant state can be controlled by the dose of DNP-D-GL administered and the interval between administration of tolerogen and challenge with DNP conjugated to an unrelated protein, such as ovalbumin (OVA).

The administration of DNP-D-GL leads to a profound (>10 fold) diminution in the number of detectable cells capable of binding DNP-GPA. Although tolerance is marked in the DNP specific pool of precursor cells, no apparent thymus derived cell tolerance is noted. Thus, immunization with DNP-GPA in complete Freund's adjuvant of guinea pigs previously subjected to a tolerance induction regime results in little or no antibody synthesis but in normal delayed skin hypersensitivity to DNP-GPA. Moreover, peritoneal exudate lymphocytes from such tolerant animals respond normally upon *in vitro* stimulation with DNP-GPA. Thus, this tolerance induction regime provides a simple method of selectively suppressing DNP-specific B cells. This will be an exceedingly valuable procedure in attempts to chemically characterize T cell receptors.

When guinea pigs are injected with sufficient DNP-D-GL to only partially suppress their subsequent response to DNP-GPA, the tolerant state is preferentially induced in the precursor cells bearing receptors of the highest affinity for DNP. This has been demonstrated by an analysis of the affinity of antibody produced by individual antibody producing cells which develop after immunization with DNP-GPA. A specific suppression in cells making high affinity antibody was observed. Similarly, the affinity of the secreted antibody and the apparent affinity of antigen binding receptors on precursor cells is lower in tolerant animals. These data imply that the induction of

tolerance in a precursor cell involves an affinity dependent antigen driven event, very likely at the cell surface.

Significance to Biomedical Research and the Program of the Institute:

The interaction of antigen with cell surface antigen-binding receptors is the key event in the specific activation or suppression of immunocompetent cells. These studies aimed at exploring, in quantitative terms, the nature of this antigen-binding event and both its immediate and long term cellular consequences should provide a firm basis for a precise understanding of the specific regulation of the immune response.

Proposed Course of Project:

These studies will be continued with particular emphasis on membrane events after antigen-binding, on the mechanism of tolerance induction and on approaches to the chemical characterization of the T cell receptor.

Publications:

Paul, W.E. and Davie, J.M.: Specificity of receptors for antigen of cells participating in a cellular immune response. In Cellular Interactions in the Immune Response. Ed. by Cohen, S., Cudkowicz, G. and McCluskey, R.T., New York, S. Karger. 1971. p. 116.

Paul, W.E. and Kask, A.M.: Structural control of immunogenicity. I. Synthesis of oligo-L-lysine peptides and their mono-ε-DNP derivatives. Immunology 21: 575, 1971.

Stupp, Y., Paul, W.E. and Benacerraf, B.: Structural control of immunogenicity. II. Antibody synthesis and cellular immunity in response to immunization with mono-ε-DNP-oligo-L-lysines. Immunology 21: 593, 1971.

Stupp, Y., Paul, W.E. and Benacerraf, B.: Structural control of immunogenicity. III. Preparation for and elicitation of anamnestic antibody responses by oligo- and poly-lysines and their DNP derivatives. Immunology 21: 595, 1971.

Paul, W.E., Stupp, Y., Siskind, G.W. and Benacerraf, B.: Structural control of immunogenicity. IV. Relative specificity of elicitation of cellular immune responses and of ligand binding to anti-hapten antibody after immunization with mono-ε-DNP-mono-L-lysine. Immunology 21: 605, 1971.

Davie, J.M. and Paul, W.E.: Receptors on immunocompetent cells. II. Specificity and nature of receptors on 2,4-dinitrophenyl guinea pig albumin- ^{125}I binding lymphocytes of normal guinea pigs. J. Exp. Med. 134: 495, 1971.

Davie, J.M., Rosenthal, A.S. and Paul, W.E.: Receptors on immunocompetent cells. III. Specificity and nature of receptors on 2,4-dinitrophenyl guinea pig albumin-^{125}I binding cells of immunized guinea pigs. J. Exp. Med. 134: 517, 1971.

Katz, D.H., Davie, J.M., Paul, W.E. and Benacerraf, B.: Carrier function in anti-hapten antibody responses. IV. Experimental conditions for the induction of hapten-specific tolerance or for the stimulation of anti-hapten anamnestic responses by "nonimmunogenic" hapten-polypeptide conjugates. J. Exp. Med. 134: 201, 1971.

Paul, W.E. and Davie, J.M.: Antigen binding receptors on lymphoid cells. Nature and specificity of receptors on various types of immunocompetent cells. Progress in Immunology 1: 638, 1971.

Davie, J.M. and Paul, W.E.: Receptors on immunocompetent cells. IV. Direct measurement of avidity of cell receptors and cooperative binding of multivalent ligands. J. Exp. Med. 135: 643, 1972.

Davie, J.M. and Paul, W.E.: Receptors on immunocompetent cells. V. Cellular correlates of the "maturation" of the immune response. J. Exp. Med. 135: 660, 1972.

Paul, W.E. and Benacerraf, B.: Antigen Recognition. In Mechanisms of Cellular Immunity. Ed. by S. Cohen and R.T. McCluskey, Wiley-Interscience, New York, in press.

Paul, W.E.: Immunogenicity: Heterogenization and Problems of Antigen Recognition. Journal of the National Cancer Institute Monograph Series. In press.

Serial No. NIAID - 141
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Specific Cellular Interactions in Immune Response

Previous Serial No.: Same

Principal Investigator: Dr. W. E. Paul

Other Investigators: Dr. J. D. Stobo
Dr. C. A. Janeway, Jr.
Dr. J. C. S. Breitner
Dr. R. Sharon
Dr. T. Yoshida
Dr. A. S. Rosenthal (LCI, NIAID)

Cooperating Units: Dr. N. Talal
VA Hospital
San Francisco, California

Dr. C. S. Henney
Johns Hopkins University Hospital
Baltimore, Maryland

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	6 9/12			6 9/12
Professional	3 9/12			3 9/12
Other	3			3

Project Description:

Objectives:

Immune responses to a variety of antigens involve the participation of two major classes of lymphocytes, the thymus derived (T) and bone marrow derived (B) lymphocyte. At the current time the nature of cellular heterogeneity within each class, the differentiation history of the cells and the nature of their interactions remain obscure. In these studies, attempts have been made with model systems to delineate the biology of T lymphocytes and their role in the activation and suppression of precursors of antibody secreting cells.

Methods Employed:

The capacity of lymphoid cells from various organs of the mouse to proliferate in vitro in response to phytohemagglutinin (PHA), concanavalin A (Con A) and other stimulants was measured by incorporation of tritiated thymidine (^{3}H-TdR) into DNA. Cellular interaction was studied in vivo by transferring spleen cells from immunized or non-immunized mice into irradiated syngeneic recipients. In vitro models for cell interaction involved the utilization of Mishell-Dutton culture systems for primary and secondary antibody responses.

Major Findings:

Con A and PHA are mitogens which stimulate only T lymphocytes. PHA is, however, a poor stimulant of thymocytes while Con A stimulates thymocytes quite actively, with the exception of thymocytes from neonatal animals which respond quite poorly to Con A. One can demonstrate within the thymus a small subpopulation of cells which are cortisone resistant, relatively mature and respond to both PHA and Con A. These observations are consistent with a maturation schema involving entry of stem cells into the thymus, subsequent acquisition of responsiveness to Con A and finally, toward the end of the maturation process within the thymus, acquisition of responsiveness to PHA. In association with these changes in mitogen responsiveness, there are changes in the concentration of the surface differentiation antigen θ. The immature Con A responsive, PHA unresponsive thymocyte has large amounts of surface θ while the more mature cortisone resistant cell which responds to both mitogens has much less surface θ.

Recent studies demonstrate that within peripheral lymphoid organs, two general subclasses of T lymphocytes can be demonstrated. One subclass responds well to both PHA and Con A, is found within the recirculating pool and is relatively abundant in lymph nodes. This cell is sensitive to anti-lymphocyte serum and to small doses of irradiation and bears a relatively high concentration of surface θ. Preliminary studies suggest that this cell is responsible for mixed lymphocyte reactivity and is equivalent to the "amplifier" cell described by Asofsky and colleagues in the study of graft-versus-host reactivity.

The other major T cell subclass responds to Con A but is relatively unresponsive to PHA, is relatively sessile and abundant within the spleen. This cell is relatively resistant to both radiation and anti-lymphocyte serum and bears considerably less surface θ than the PHA responsive cell. It appears to be the effector cell in in vitro T cell-mediated cytotoxicity reactions. In addition, this cell is required for antigen-stimulated in vitro DNA synthesis and is equivalent to the cell which Asofsky, et al. term the precursor of the effector cell in graft-versus-host reactions.

At the current stage of investigation on this problem, it is not yet certain whether the two types of T lymphocytes represent two independent lines which are undergoing parallel differentiation or, alternatively, two stages in the differentiation history of a single cell line.

In the course of studies on T lymphocytes, it has been observed that some splenic lymphocytes lack the surface markers characteristic of both the T and B cell lines. Thus, from 5 to 10% of spleen lymphocytes from normal mice of various strains lack the θ antigen and have either no surface immunoglobulin or too little to be detected by radioautography with ^{125}I-anti-κ antibody. These θ^-; Ig^- cells are not θ^- thymus derived lymphocytes as demonstrated by the finding that in chimeric C57BL/6 animals in which the T cells are derived from a (C57BL/6xBALB/c)F_1, the θ^-, Ig^- cells lack the histocompatibility antigens of the BALB/c. Moreover, the θ^-; Ig^- cell is not a hematopoietic stem cell. Thus, enrichment for θ^-; Ig^- cells does not enrich for exogenous colony forming unit content. Furthermore, θ^-; Ig^- cells lack peroxidant enzymes characteristic of more mature precursor cells. These θ^-; Ig^- cells have the typical appearance of small lymphocytes by electron microscopy. Labeling studies demonstrate a relatively slow turnover rate.

Although the function of θ^-; Ig^- lymphocytes is presently uncertain, a highly provocative finding is that these cells are markedly increased in frequency in NZB, NZW and (NZBxNZW)F_1 mice. These latter mice have a variety of autoimmune disorders and the relatively high frequency of θ^-; Ig^- lymphocytes may be important in the pathogenesis of their disease. These NZ animals have, in gross terms, normal numbers of T lymphocytes but the frequency of B (Ig^+) lymphocytes is reduced at all ages tested beginning at birth and extending to the age of one year.

In parallel with these studies of lymphocyte biology, studies of cellular interactions are also proceeding. One of the key issues in the understanding of cell-cell interactions is the relative specificity of the two cell types. In general, the B cell, which is the precursor of the antibody secreting cell, may be specific for haptenic groups, for determinants on carrier molecules as well as for so-called "link" determinants involved both hapten and carrier group. T cells, on the other hand, do not seem to demonstrate functional specificity for haptenic determinants. This discrepancy in specificity may be of profound importance in the understanding of the T cell function or it may be a simple random event. A recent report by Iverson that a hapten specific "helper" effect was demonstrable was therefore important to evaluate for, if due to specific T cell function, it would render less likely the concept that profound specificity differences exist between B and T cells. An attempt to repeat the Iverson experiment was thus made. The system employed involves the anti-idiotype response of BALB/c mice to the γG myeloma protein LPC-1 which is derived from BALB/c. BALB/c mice normally fail to make any appreciable anti-idiotype response to LPC-1 or to a DNP derivative of LPC-1. However, preimmunization of mice with DNP-ovalbumin does prepare them for a significant anti-idiotype response to DNP-LPC-1 but not to LPC-1. This, then, is a hapten specific helper effect and is consistent with Iverson's report. The effect, however, is not due to DNP specific T cells as transfer of spleen cells from animals primed to DNP-OVA does not prepare the recipient for a response to DNP-LPC-1. On the other hand, transfer of serum from the DNP-OVA primed donor to a syngeneic recipient allows the latter animal to respond to DNP-LPC-1. This effect is mediated by anti-DNP antibody as demonstrated by

the capacity of DNP or anti-immunoglobulin coupled to agarose beads to remove the 'helper" activity from the serum. Thus, serum antibody may have an important role in increasing immunogenicity of weak antigens and no definitive evidence for existence of hapten-specific T cells has yet been obtained.

The final portion of the studies in this project are those aimed at elucidating a mechanism for this interaction. Two model systems have been selected. In the first, the capacity of T cell stimulants to affect the m agnitude of primary in vitro anti-sheep erythrocytes (SRBC) antibody responses was measured. The addition of either Con A, PHA or pokeweed mitogen at stimulatory or sub-stimulatory concentrations has profound suppressive activity on the magnitude of a simultaneous primary anti-SRBC response. Indeed, as little as 3 nanograms of Con A per ml of culture medium cause a significant depression of the SRBC response. At the current time, the locus of action of Con A in this suppressive situation is being sought.

The second model system is that of macrophage inhibition factor (MIF). MIF is the prototype biologically active substance produced by T lymphocytes as a result of antigenic stimulation. It has recently been reported that MIF is specific; that is, in the absence of antigen MIF lacks its functional capacity to inhibit migration of macrophages out of a capillary tube. This would suggest that functional lymphokine-antigen complexes exist and might play an important role in cell cooperation. A detailed study of the antigen-dependence of MIF was thus made. Lymph node cells from guinea pigs immunized with DNP-proteins were stimulated in vitro with the immunizing antigen. At the termination of the culture period, antigen was completely removed from the MIF containing supernatant by affinity chromatography on anti-DNP agarose beads. These antigen-free MIF preparations had full activity. Re-addition of antigen caused no increase in the activity of MIF. These and similar experiments provide no foundation whatever for the claim that antigen-specific lymphokines exist and thus render somewhat less likely the possibility that an antigen-specific soluble factor is involved in the cooperative interactions of B and T cells.

Significance to Biomedical Research and the Program of the Institute:

A key to the fuller understanding of the activation of both T and B lymphocytes and to our ability to precisely regulate immune responses is a delineation of the full complexity of lymphocyte biology and the mechanisms of interaction between the major lymphocyte classes. These studies represent considerable steps toward the fulfillment of these major goals.

Proposed Course of Project:

Further detailed functional studies of T cell subsets and of θ^-; Ig^- cells will be undertaken. To adequately achieve these goals, improved methods for obtaining pure populations of these various cell types are required. In addition, further efforts to delineate the mechanism of T-B interaction are planned, utilizing both in vivo and in vitro models.

Publications:

Benacerraf, B., Katz, D.H. and Paul, W.E.: Cell cooperation in the antibody response to hapten-protein conjugates. VIth International Symposium on Immunopathology. In Miescher, P.A. (Ed.): Immunopathology. Basel, Switzerland, Schwabe & Co., 1970, pp 24-34.

Katz, D.H., Paul, W.E. and Benacerraf, B.: Carrier function in anti-hapten antibody response. V. Analysis of cellular events in the enhancement of antibody responses by the "allogeneic effect" in DNP-OVA primed guinea pigs challenged with a heterologous DNP-conjugate. J. Immunol. 107: 1319, 1971.

Paul, W.E., Katz, D.H. and Benacerraf, B.: Augmented anti-SIII antibody responses to an SIII-protein conjugate. J. Immunol. 107: 685, 1971.

Benacerraf, B. and Paul, W.E.: Cellular organization of the immune system: Overview and synthesis. In Cohen, S., Cudkowicz, G. and McCluskey, R.T. (Eds.). Cellular Interactions in the Immune Response. New York, S. Karger, 1971, pp 298-307.

Stobo, J.D., Rosenthal, A.S. and Paul, W.E.: Functional heterogeneity of murine lymphoid cells. I. Responsiveness to and surface binding of concanavalin A and phytohemagglutinin. J. Immunol. 108: 1, 1972.

Stobo, J.D. and Rosenthal, A.S.: Biologically active concanavalin A complexes suitable for light and electron microscopy. Exptl. Cell. Res. 70: 445, 1972

Stobo, J.D. and Paul, W.E.: Functional heterogeneity of murine lymphoid cells. II. Acquisition of mitogen responsiveness and of antigen during the ontogeny of thymocytes and "T" lymphocytes. Cellular Immunol. In press.

Yoshida, T., Janeway, C.A., Jr. and Paul, W.E.: Activity of migration inhibitor factor in the absence of antigen. J. Immunol. In press.

Stobo, J.D. and Paul, W.E.: Heterogeneity of peripheral T lymphocytes. Fourth International Conference on Lymphatic Tissue and Germinal Centers in Immune Reactions. Plenum Press. In press.

Stobo, J.D.: Phytohemagglutinin and concanavalin A: Probes for murine "T" cell activation and differentiations. Transplantation Reviews. In press.

Serial No. NIAID - 142
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Genetic Control of Immune Response

Previous Serial No.: 142

Principal Investigator: Dr. Ira Green

Other Investigators:

Project Subtitle I:	Dr. Harry Bluestein	
	Dr. Baruj Benacerraf	
Project Subtitle II:	Dr. William E. Paul	
	Dr. Baruj Benacerraf	
Project Subtitle III:	Dr. Joseph Davie	
	Dr. William E. Paul	
Project Subtitle IV:	Dr. Ethan Shevach	
	Dr. William E. Paul	

Cooperating Units: Harvard Medical School, Department of Pathology
25 Shattuck Street, Boston, Massachusetts

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1 6/12			1 6/12
Professional	1			1
Other	6/12			6/12

Project Subtitle I: Genetic Control of Immune Responsiveness to Copolymers of L-glutamic acid-L-alanine (GA), L-glutamic acid and L-tyrosine (GT) to the terpolymer L-glutamic acid, L-alanine and L-tyrosine (GAT)

Project Description:

Objectives:

The ability of guinea pigs to respond to a simple synthetic poly amino acid antigens (poly-L-lysine) has been shown to be controlled by the presence of a autosomal dominant gene (IR genes). The IR gene in turn has been shown to be linked to the presence of the major strain 2 histocompatibility locus (H). In the present study we asked whether other IR genes linked to H type

could be demonstrated in guinea pigs and to determine whether these new IR genes were linked to the prototype PLL gene.

Methods Employed:

Strain 2, strain 13 and $(2x13)F_1$ x 2, $(2x13)F_1$ x 13 guinea pigs, as well as NIH multipurpose guinea pigs, NIH Hartley guinea pigs and Camm Hartley guinea pigs were immunized with GA, GT, GAT and DNP-PLL in CFA. Delayed hypersensitivity (DS) was assayed by skin testing with appropriate amounts of these antigen; antibody responses were measured by Farr techniques using ^{125}I conjugates of these antigens. In vitro lymph node cell stimulation by these antigens were performed using lymph node lymphocytes and pulsing the cells with ^{3}H thymidine after 48-72 hours in culture.

Determination of histocompatibility (H) type was assayed using lymph node cells as target and cytotoxic 2 anti 13 or 13 anti 2 sera in the presence of complement. The degree of cytotoxicity was measured using ^{51}Cr release.

Major Findings:

All guinea pigs respond to the terpolymer GAT. They develop both D.S. and anti-GAT antibody. However, only strain 2 guinea pigs responded to GA and only strain 13 guinea pigs respond to GT. A variable proportion of Hartley guinea pigs respond to GA or GT. When Hartley guinea pigs were immunized with DNP-PLL and GA, most guinea pigs that responded to DNP-PLL also responded to GA and vice versa. However, there were some guinea pigs that were responsive to GA and unresponsive to DNP-PLL and a very rare animal that was DNP-PLL+ and GA-. When Hartley guinea pigs were immunized with DNP-PLL and GT, the reverse was observed. That is, guinea pigs that were DNP-PLL+ were only rarely GT+ and GT+ guinea pigs were only rarely DNP-PLL+. NIH multipurpose guinea pigs had a high frequency of response to GT and a very low frequency of response to DNP-PLL. To ascertain whether these immune responses was linked to the H locus of these guinea pigs, suitable backcross animals were immunized with either GA and GT and the presence of the strain 2 or strain 13 H locus determined by ^{51}Cr release techniques using cytotoxic anti-H antibody. This result was correlated with the presence or absence of immune responses in these backcross animals. Thus, to evaluate the GA response, $(2x13)F_1$ x 13 backcross animals were immunized with GA and their immune response measured and presence or absence of strain 2 H antigen was also determined. In every case animals that possessed the strain 2 H locus were GA responders; backcross animals lacking the strain 2 H locus failed to respond to GA. For this type of analysis in the case of GT, $(2x13)F_1$ x 2 backcross animals were used. Here again, only those animals possessing the major strain 13 H locus responded to GT; animals lacking this locus failed to respond.

From this data as well as from information obtained from results in random bred Hartley and NIH multipurpose animals it appeared that GA and DNP-PLL IR genes were not identical but were closely linked on the same

chromatid.

However it appeared that the GT IR gene and the PLL gene were alleles or pseudoalleles. If this prediction was correct, then $(2x13)F_1$ animals possessing both the GT gene and PLL gene on separate chromatids when mated with NIH multipurpose guinea pigs that were selected to be PLL- and GT- should result in progeny that were either GT+ or DNP-PLL+. In seven offspring of such matings this prediction was borne out, animals were either DNP-PLL+ or GT+, never both.

Next, we examined the H type of random bred guinea pigs capable of responding to GT to see whether these animals also possessed the strain 13 H locus. All GT+ animals had the 13 H locus and most of the GT negative animals did not have the 13 locus. However the lymph node cells from some GT- animals were lysed but to a lower degree by 2 anti-13 sera and complement. If this sera was absorbed with cells of these GT negative animals, the sera failed to lyse the lymph node cells of other animals of this type but when tested against the cells of GT responder animals caused definite but diminished cytotoxicity. These studies, together with the results in the F_1x2, backcross animals suggest that there are two separate 13 specificities, one of which is very closely linked to the GT IR gene and the other one, although on the same chromatid is somewhat more distant.

Finally, we wished to investigate whether the presence of the GT and GA immune response gene would influence the response to the more complex terpolymer GAT. As mentioned earlier, GAT is an immunogen for all guinea pigs. It should be noted that there is no cross reaction in guinea pigs between GA and GT as measured either by antibody or cellular immunity. If Hartley guinea pigs are immunized to DNP-PLL and GAT and then skin tested with GA, D.S. reactions to GA were more frequent in the DNP-PLL positive animals than in the DNP-PLL negative animals. Since the DNP-PLL is closely linked to the GA gene these results indicate that the presence of GA IR gene significantly influences the specificity of the D.S. response to GAT. If the specificity of the anti GAT antibodies are examined in these animals by using binding of radioactive GAT and GA as ligands both GA skin test positive and negative animals produced antibody to GAT, however, only the sera of GA positive animals could find GA. Similar observations were also made in 2 and 13 animals immunized with GAT. Here only the sera of GA positive strain 2 animals could bind GA; sera of strain 13 did not bind GA. The anti-GAT binding ability of the sera of 2 and 13 animals immunized with GA were however very similar. These studies indicate that the fine specificity of antibodies to more complex antigens can be influenced by presence of IR genes which presumably function chiefly in thymus derived cells (T). In simplest terms IR genes in T cells may direct the presentation of the antigen to B cells.

Significance to Biomedical Research and the Program of the Institute:

The availability of several different antigens, controlled by immune response genes and linked to different H antigens will provide a powerful tool to further probe the meaning and significance of IR genes--H antigen

linkage. Indeed (see next section) these studies are not being carried out.

Since the immune response to more complex antigens are also mediated by H linked IR gene function and since H linkage has been detected in a variety of human disease progress in an understanding of IR function may provide part of the answer as to why certain human disease are associated with certain H type.

Project Subtitle II: Genetic Control of Immune Response to 2,4 dinitrophenyl conjugates of guinea pig albumin (DNP-GPA)

Project Description:

Objectives:

During the course of studies of response to low doses of DNP-bovine serum albumin (DNP-BSA) in guinea pig it was observed that there was a marked difference in the response of inbred strain 2 and 13 guinea pigs to 1 μg of DNP-GPA guinea pig albumin containing 6 moles of DNP per mole of protein (DNP_6GPA). In contrast to the results observed with DNP-BSA, where strain 2 pigs respond and strain 13 animals fail to respond, strain 13 animals immunized with 1 μg DNP_6GPA produced high levels of anti-DNP antibody and strain 2 animals failed to respond. This same difference was also observed with DNP_{33}GPA. To determined whether these differences in response could be attributed to the hapten part of the antigen or to the carrier part of the antigen the following experiments were performed. Immunization of 2 and 13 pigs with DNP hapten on an unrelated carrier, glucose oxidase demonstrated that at several doses of immunogen both 2 and 13 animals made an equivalent anti-DNP antibody response. This suggested that the carrier was, indeed, important. Moreover, when 2 and 13 guinea pigs were immunized with 1 μg of pipsyl GPA, pipsyl being only very poorly cross reactive with dinitrophenyl hapten, strain 13 animals made significant levels of anti-pipsyl antibodies, whereas strain 2 animals failed to produce antibody. Finally, in the usual DNP protein conjugates the dinitrophenyl hapten is attached to the ε-amino groups of lysine. The dinitrophenyl group can, however, be attached to the carboxyl groups of glutamic and aspartic acid. When strain 2 and 13 guinea pigs are immunized with this material at 1 μg, again 13 respond by producing anti-DNP whereas strain 2 animals fail to respond. This strongly suggests the recognition carrier perhaps in unmodified form, is specifically controlled by the IR gene. Finally we wished to see whether the ability of inbred guinea pigs to respond to DNP-GPA was linked to the major strain 13 H locus. Therefore $(2\times13)F_1$ x 2 backcross animals were immunized with 1 μg DNP_6GPA and the ability of these animals to make anti-DNP antibody was correlated with their possession of the major strain 13 H locus as determined with a strain 2 anti-strain 13 H sera (see previous section). Fourteen such animals were examined. Seven produced antibody and had the strain 13 specificities and 7 animals failed to respond and lacked the strain 13 specificities. Thus the ability to respond to limiting doses of DNP_6GPA is linked to the major strain 13 H locus of inbred guinea pigs.

Significant to Biomedical Research and the Program of the Institute.

Here we have demonstrated that the ability to respond to an autologous carrier can be determined by the presence of Ir genes linked to H types. Since a variety of modifications of the autologous carrier did not alter its immunological behavior, we conclude that there is an IR gene for unmodified GPA which however cannot usually be detected. Only by breaking tolerance with modified GPA however can the operation of such an IR gene be detected.

The ability of an organism to respond to self proteins may therefore in part be determined by presence or absence of IR genes. The implications of these findings for pathogenesis of "autoimmune disease" are obvious.

Project Subtitle III: Genetic control of immune response of inbred guinea pigs to 2,4-dinitrophenyl guinea pig albumin (DNP-GPA). Frequency of antigen binding lymphocytes and avidity of antibody secreted by plaque forming cells.

Project Description:

Objectives:

In the previous section we described the fact that the immune response to DNP-GPA was controlled by an Ir gene linked to the major strain 13 H locus. To more precisely delineate the nature of the defect in the non responder strain 2 animals, the number of antigen binding cells prior to immunization as well as the affinity of the antibodies produced in strain 2 and 13 animals after immunization was determined. $(2\times13)F_1$ x 2 backcross animals were also examined prior to immunization for the number of antigen binding cells and this was correlated with their later response to 1 μg DNP_6GPA.

Methods Employed:

Animals were immunized with 100 μg or 1 μg DNP_6GPA in CFA. Lymph nodes were removed at later intervals and the teased cells studied by Jerne plaque techniques for the presence of direct and indirect anti DNP antibody secreting cells. The affinity of the antibody secreted was determined by the ability of various concentrations of ε-DNP-lysine in the agar to inhibit plaque formation. The animals were bled and the affinity of the anti-DNP antibody was measured by the antigen binding capacity of the serum at 2 ligand concentrations using a Farr procedure.

To determine the number of antigen binding cells present in the lymph nodes of F_1 x 2 backcross animals prior to immunization, the animals were first injected with adjuvant and saline in one rear foot pad. 2 weeks later the node was removed, teased and cells exposed to ^{125}I DNP-GPA. After washing the cells were removed and examined by radioautography.

An aliquot of these cells was also used for determination of H type using a ^{51}Cr release cytotoxicity technique.

The animals were subsequently immunized with 1 μg DNP_6GPA and their antibody response correlated with the number of antigen binding cells present prior to immunization and presence or absence of strain 13 H type.

Major Findings:

When strain 2 and 13 guinea pigs are immunized with 1 μg DNP_6GPA only strain 13 animals develop direct and indirect plaque forming cells (PFC). When 100 μg DNP_6GPA is used both animals develop PFC, but the number of PFC is lower in strain 2 animals and their response is delayed as compared to strain 13 animals. Nevertheless, at 28 days the affinity of the antibody secreting cells in strain 2 and 13 animals is identical as is the affinity of the serum anti-DNP antibody.

Next the number of antigen binding cells was determined in $(2x13)F_1$ x 2 backcross animals prior to immunization and this data was correlated with the subsequent antibody response of these animals after immunization with 1 μg DNP_6GPA. There was no correlation between the number of antibody binding cells and the subsequent response. There was, however, a positive correlation as observed previously between the anti DNP antibody response and the presence of the strain 13 H antigen.

Significance to Biomedical Research and the Program of the Institute:

This study established that the antigen binding cells, which are the anti DNP antibody forming cell precursors, are normal in number in animals incapable of producing anti DNP antibody when immunized with limiting doses of DNP_6GPA. This, therefore, strongly suggests that the defect in the non responder strain 2 animals is in the T cell. The development and availability of an immune system under genetic control and which are also linked to H type will allow for a concerted attack on the mechanisms of genetic regulation and T cell function. The fact that these present studies deal with an Ir gene to a self protein may have special relevance to problems of autoimmune disease.

Project Subtitle IV: Effect of anti-histocompatibility antisera on immune response gene function

Project Description:

Objectives:

A number of immune response genes have been identified in inbred guinea pigs which control responses to specific antigens. In strain 2 animals one gene controls responsiveness to Poly-L-lysine (PLL), poly-L-arginine (PLA), a copolymer of L-glutamic acid and L-lysine (GL), protamine, and hapten conjugates of these polypeptides such as DNP-PLL. In strain 13 animals a gene which controls responsiveness to a random copolymer of L-glutamic acid and L-tyrosine (GT) has recently been described. These specific immune response genes are closely linked to the major histocompatibility genes. These animals

in random bred animals the PLL gene cannot be dissociated from the gene controlling strain 2 specificity. One way of examining the relationship between IR genes and immune recognition is to test the effect of specific anti-histocompatibility sera on antigen induced lymphocyte stimulation.

Methods Employed:

Strain 2 animals were immunized with DNP-GL; strain 13 with GT and (2x13)F_1 with both DNP-GL + GT. The effect of anti-histocompatibility sera on antigen induced blast transformation was measured by culturing the cells either in normal serum or in specific anti histocompatibility sera obtained from animals which had been immunized with lymphoid cells of the other strain.

Major Findings:

The 13 anti 2 serum inhibited the response to DNP-GL in strain 2 animals and the 2 anti 13 inhibited the response to GT in strain 13 animals. However, both also inhibited to a similar extent the response to PPD, an antigen which is not known to be under genetic control. When these antisera were used with F_1 cells in culture, the 13 anti 2 inhibited the DNP-GL response, but not the GT, and the 2 anti 13 inhibited the GT response, but not the DNP-GL. Thus in F1 animals these alloantisera specifically block the response to the antigens controlled by the immune response gene which is linked to the major histocompatibility antigens recognized by the alloantisera.

Significance to Biomedical Research and the Program of the Institute:

The mechanism of action of the immune response genes is now known. Our studies demonstrate that alloantisera specifically block the mechanism of antigenic recognition of genetically controlled antigens by the thymus derived lymphocytes. It is therefore likely that the immune response gene product is present on the surface of the T lymphocyte and is either identical to histocompatibility antigen or physically closely related to histocompatibility antigen on the cell surface. The third possibility is that the inhibitory activity of our alloantisera is independent of its anti histocompatibility activity and it is directed against a specific immune response gene receptor on the cell surface.

The nature of the IR gene product is of crucial importance to our understanding of the function of the normal immune response. In addition, since susceptibility to a number of malignant and auto-immune diseases has been linked to histocompatibility type, the IR genes may play an important role in susceptibility to a variety of diseases.

Proposed Course of Project:

These studies are being continued and the relationship between the IR gene product and histocompatibility antigen are being explored.

Publications:

Bluestein, H.G., Green, I. and Benacerraf, B.: Specific immune response genes of the guinea pig. I. Dominant genetic control of immune responsiveness to copolymers of L-glutamic acid and L-alanine, GA and L-glutamic acid and L-tyrosine, GT. JEM 134: 458, 1971.

Bluestein, H.G., Green, I. and Benacerraf, B.: Specific immune response genes of the guinea pig. II. Relationship between the "PLL Gene" and the genes controlling immune responsiveness to GA and GT in random-bred Hartley guinea pigs. JEM 134: 471, 1971.

Ellman, L. and Inman, J., Green, I.: Strain difference in the immune response to hydralazine in inbred guinea pigs. Clinical and Exp. Immunol. 9: 927, 1971.

vanBoxel, J., Steinberg, A. and Green, I.: A study of the antibody mediated immune responses to ribonucleic acids in guinea pigs. J. Immunology 108: 446, 1972.

Bluestein, H.G., Ellman, L., Green, I. and Benacerraf, B.: Specific immune response genes of the guinea pig. III. Linkage of the GA and GT immune response genes to histocompatibility genotypes in inbred guinea pigs. JEM 134: 1529, 1971.

Bluestein, H.G., Green, I. and Benacerraf, B.: Specific immune response genes of the guinea pig. IV. Demonstration in random-bred guinea pigs that responsiveness to a copolymer of L-glutamic acid and L-tyrosine is predicated upon the possession of a distinct strain 13 histocompatibility specificity. JEM 134: 1538, 1971.

Bluestein, H.G., Green, I., Maurer, P.H. and Benacerraf, B.: Specific immune response genes of the guinea pig. V. Influence of the GA and GT immune response genes on the specificity of cellular and humoral immune responses to a terpolymer of L-glutamic acid, L-alanine and L-tyrosine. JEM 135: 98, 1972.

Green, I., Paul, W.E. and Benacerraf, B.: Histocompatibility linked genetic control of the immune response to hapten guinea pig albumin conjugates in inbred guinea pigs. J. Immunol. in press.

Davie, J.M., Paul, W.E. and Green, I.: Genetic control of the immune response of inbred guinea pigs to 2,4-dinitrophenyl guinea pig albumin. Frequency of antigen-binding lymphocytes and avidity of antibodies secreted by plaque forming cells. J. Immunol. in press.

Serial No. NIAID - 143
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Lymphocyte Interactions, Receptors and Functions

Previous Serial No.: 143

Principal Investigator: Dr. Ira Green

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Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	4 6/12			4 6/12
Professional	3 6/12			3 6/12
Other	1			1

Project Subtitle I: Receptors for Antigen-Antibody and Antigen-Antibody Complexes on Lymphoid Cells

Project Description:

Objectives:

Cells involved in the immune response can be functionally characterized by the presence of different membrane antigens and receptors. In the mouse, thymus derived (T cells) bear the θ isantigen; bone marrow derived (B cells) can be identified by the presence of easily detectable surface immunoglobulin. B cells also bear a receptor for antigen-antibody-complement complexes (EAC). Monocytes and macrophages, the third cell type involved in the immune response also bear a receptor for EAC, but in addition bear a receptor for antigen-antibody complexes (EA). The presence or absence of these various receptors on cells of unknown type may provide information as to their origin. In the present study malignant lymphoid cells from both mice and humans were examined for the presence of these various receptors.

Methods Employed:

In order to detect the receptor for antigen-antibody-complement complexes (EAC), the lymphoid cell population to be studied is incubated with red cells that have been coated with anti-red cell antibody of the IgM type and mouse serum as the source of complement. The precentage of cells binding these complexes is determined by counting in a hemacytometer. The presence of the receptor for antigen antibody complexes (EA) is detected in a similar fashion with red cells that are coated with anti-red cell antibody of the IgG type. The mouse leukemias and lymphomas were also examined for the presence of the θ isoantigen and surface immunoglobulin by standard immunofluorescent and cytotoxicity techniques.

Major Findings:

Twenty-one mouse leukemias and lymphomas were examined for the presence of the EAC and EA receptors. None of the tumors studied bore the EAC receptor of the B lymphocyte. One tumor could be characterized as a monocyte by the presence of the monocyte EAC and EA receptors; two of the tumors bore the monocyte EA receptor alone. It was also demonstrated that the binding of the IgG EA complex to monocyte-like tumors and to normal monocytes could be inhibited only by mouse myeloma of the γ_1 and γ_{2b} subclasses of mouse IgG.

Twenty-four murine leukemias and lymphomas and eight plasma cell tumors were investigated for the presence of the θ isoantigen and determinants of the κ chain. Twelve of the leukemias studied have the θ isoantigen and are of T cell origin; one tumor bore κ light chain and is presumably of B cell origin although it did not bear the lymphocyte receptor for EAC. Eleven of the tumors studied had no detectable θ or κ determinants. Four of the eight plasma cell tumors were also shown to have surface κ determinants.

The percentage of normal peripheral blood lymphocytes in human blood which bear the receptor for EAC was shown to range from 3-12%. The cells of 8 patients with chronic lymphatic leukemia had from 25-100% EAC binding cells in peripheral blood; none of these cells bound the EA receptor and hence chronic lymphatic leukemia cells are probably of B cell origin. Neither the EAC or EA receptors could be demonstrated in the cells from patients with acute lymphatic leukemia, acute myelogenous leukemia, or chronic myelogenous leukemia.

Nineteen human lymphoblastoid cell lines were also examined for the presence of these receptors. 12/19 bore the EAC receptor and are probably of B cell origin. 2/19 cell lines possessed both the EAC and EA receptor and thus resemble the monocyte. 5/19 cell lines had no detectable receptors for EAC or EA.

Significance to Biomedical Research and the Program of the Institute:

Both the receptors for EAC and EA are easily detectable on lymphoid or monocytoid cells of unknown origin. The presence of these various receptors, surface immunoglobulin, or the isoantigen in the case of murine cells, provides an important clue as to the cell type of origin of these malignant populations of cells. Furthermore, the availability of large numbers of uniform malignant cells with receptors characteristic of normal cells offers the opportunity for detailed study and characterization of these receptors. The complement and immunoglobulin receptors may play a role in antibody formation especially in antigen processing and cellular localization within lymph nodes. The further detailed study of the lymphocytes and monocytes from patients with malignant, infectious and immunologic diseases for both qualitative and quantitative deficiencies of the immunoglobulin and complement receptors as well as the B or T cell origin of their lymphocytes should prove rewarding.

Proposed Course of Project:

We will continue to examine both murine and human malignant lymphoid cells for the presence of these receptors. Hopefully, the studies can be expanded to include patients with immunologic deficiency and autoimmune diseases.

Project Subtitle II: General Biologic Properties of the L2C Cell

Project Description:

Objectives:

Lymphoid cells that participate in the immune response can be divided into two functional subtypes, the thymus derived (T) cell or the bone marrow derived (B) cell. We have studied the L2C leukemia cell of the guinea pig in order to determine if this homogeneous population of lymphocytes belongs to one of these functionally distinct subpopulations. We have also used the L2C leukemia cell to prepare a heterologous anti-lymphocyte serum which is specific for the thymus derived cell population.

Methods Employed:

The L2C cell was examined in the living state by the immunofluorescent technique for the presence of surface immunoglobulin. The presence of the lymphocyte receptor for antigen-antibody complexes was determined by incubating L2C cells with red cells coated with anti-RBC antibody and complement. The ability of the L2C cell to respond to mitogen under standard tissue culture conditions was also examined.

A heterologous antiserum to guinea pig thymocytes was prepared in rabbits and burros. The antiserum was then absorbed with L2C cells. A number of functional studies were carried out to show that the antiserum was then specific for T cells and did not kill B cells. These studies included mass killing experiments with examination of those living cells remaining for the presence of surface immunoglobulin by the immunofluorescent technique, the response of these cells in cultures to antigen and non specific mitogens, and the ability of the cells to form direct plaques in the Jerne plaque assay.

Major Findings:

1. L2C cell - Immunofluorescent studies demonstrated that the L2C cell had surface immunoglobulin of only the $\gamma 2$ class. It was not stained by the anti γ_1, anti γM or anti γA reagents. The L2C cell also secreted small quantities of only γ_2 immunoglobulin as determined after short term culture with radioactive HC lysine. 75-80% of L2C bear the lymphocyte receptor for EAC. When the L2C cell is cultured in the presence of mitogens which are known to stimulate only T lymphocytes, no stimulation of the L2C cell is seen. Thus, the L2C cell appears to be of B lymphocyte origin.

2. The specific antiserum directed toward thymocytes and thymus derived peripheral T lymphocytes was prepared by absorption of a heterologous antiserum with L2C cells selectively killed only T lymphocytes. Marked inhibition of the response of lymph node cells to antigen and mitogen was produced by treatment with the antiserum. Both of these functions are probably T lymphocyte functions. The ability of spleen cells from an immune animal to form direct plaques in the Jerne plaque assay was not impaired. Since the plaque forming cells are derived from B lymphocytes, it is likely that the antiserum does not affect B lymphocyte function.

Significance to Biomedical Research and the Program of the Institute:

Even though the L2C cell is a leukemic cell, it has been proven to be an excellent model of the normal bone marrow derived lymphocyte. The availability of a large number of pure B lymphoid cells should provide a useful tool for the study of cellular receptors of lymphoid cells, the precise manner in which immunoglobulins are attached to cell membranes, the nature of the complement receptor, and the distribution of histocompatibility antigen on cell surface.

The anti-T cell antiserum should be a useful test to examine the in vivo and in vitro manifestations of delayed hypersentsitivity. The approach used in this study could be applied to other species, particularly man, where a pure population of B lymphocytes, chronic lymphatic leukemia is also available for study and use in absorption experiments.

Proposed Course of Project:

The L2C cell will continue to be used as a model in guinea pigs for the bone marrow derived lymphocyte population in an attempt to find other morphological and functional properties of that subset of lymphocytes. The anti-T cell is being used in a number of studies to define the role of the thymus derived lymphocyte both in in vivo and in vitro immunological reactions.

Project Subtitle III: Demonstration of Thymus-derived Cell Surface Antigens on Various Guinea Pig Lymphoid Cell Populations by a Micro-Immune Adherence Technique

Project Description:

Objectives:

The presence of thymus-derived lymphocytes (T cells) in various guinea pig lymphoid cell populations is well established on a functional basis both in vivo (delayed hypersensitivity) and in vitro (antigen stimulated DNA synthesis and migration inhibition factor production). Recently a heterologous antiserum against guinea pig thymus-derived (ATDC) was prepared and has been shown to be cytotoxic to T cells. Immune adherence (IA) is a highly sensitive and specific immunological technique for the detection and quantitation of cell surface antigens. Employing a micro-IA technique using ATDC, we studied different lymphoid cell populations for the presence of cell surface antigenic specificities of T cells.

Methods Employed:

The IA phenomenon involves the binding of indicator primate erythrocytes by a C'3 receptor to antigen-antibody-complement complexes. Target guinea pig lymphoid cells from the thymus or lymph node or purified peritoneal exudate lymphocytes were coated with a burro ATDC and human or guinea pig complement (C') and then exposed to human indicator erythrocytes. The reaction mixture was examined microscopically for the percent of lymphoid cells that had formed rosettes with the erythrocytes. To demonstrate that the rosetted lymphoid cells were indeed T cells, lymph node cells were coated with ATDC and C' and after washing, labeled with fluoresceinated anti-guinea pig Fab antiserum (which has been shown to label only bone marrow derived lymphocytes (B cells)), and then reacted with indicator erythrocytes. This reaction mixture was examined under ultra-violet microscopy for the percent of rosetted lymphoid cells that were simultaneously fluorescent.

Major Findings:

We have shown that the micro-IA assay can detect antigens specific for T cells on the surface of some thymocytes, lymph node cells and purified peritoneal exudate lymphocytes. The thymus contained a higher frequency of IA positive cells as compared to lymphoid cells found in lymph node or purified peritoneal exudate cells. Furthermore, by using progressive dilutions of ATDC or C' in micro IA assays we have shown that T cells present in lymph nodes have fewer specific T cell antigenic determinants than do T cells present in the thymus. By the simultaneous IA and fluorescent anti-Fab staining techniques we have shown that the burro ATDC is highly specific for T cells in comparison to a burro anti-lymphocyte antiserum.

Significance to Biomedical Research and the Program of the Institute:

Clinically the use of anti-lymphocyte (ALS) for suppression of host rejection of homologous organ transplants is well recognized. Graft rejection is widely held to be a result of cell mediated immunity and this phenonenon has been shown to be a T cell function. Presumably then, an ALS that is specific for T cells would be preferable for purposes of immunosuppression to one which has broader specificities. The methodology used to prepare and evaluate the specific ATDC in these studies can be used as a general method for preparing antisera highly specific for human T cells. Recently, it has been possible to identify and classify certain human lymphoid cells as B cells (e.g., chronic lymphatic leukemia cells). These populations could be used to render anti-human lymphocyte sera specific for T cells.

Project Subtitle IV: Studies on the Effect of a Mild, Transient Graft-Versus-Host Reaction on Delayed Hypersensitivity, Antibody Production and Inflammatory Response

Project Description:

Objectives:

It has previously been shown that the transfusion of immunocompetent strain 13 guinea pig lymphoid cells into hapten-carrier primed strain 2 guinea pigs produces a spontaneous rise in the anti-hapten antibody titer in the recipient and allows the recipient to mount a secondary antibody response to the hapten coupled to a different carrier. This phenomenon of the effect of a mild, transient graft vs. host reaction (GVH) has been called the "allogeneic effect". It has been demonstrated that transfusion of strain 13 lymphoid cells into strain 2 guinea pigs that have earlier been given L_2C leukemia (a transplantable strain 2 guinea pig acute lymphatic leukemia) produces prolonged survival in the recipients. Also, it has been shown that these recipients have not manifested a detectable antibody response to the L_2C leukemia as a result of the "allogeneic effect". The purpose of the current studies was to investigate the effects of transfusion of allogeneic lymphoid cells on the recipient's delayed hypersensitivity (DH) capacity, antibody production and inflammatory response.

Methods Employed:

Strain 2 guinea pigs were primed with dinitrophenylated ovalbumin (DNP-OVA) or DNP-bovine serum albumin (DNP-BSA) intraperitoneally. Three weeks later they received strain 13 guinea pig lymph node and spleen cells intravenously. Six or thirteen days later the animals were skin tested with the priming antigen and 24 hours later the skin test site was examined and compared to the skin test site on primed strain 2 animals that had not received strain 13 cells. The in vitro production of migration inhibition factor (MIF) in response to the antigen of priming by mineral oil induced peritoneal exudate cells was determined at 6 and 13 days after the receipt of allogeneic cells. Sera were collected at various intervals after the receipt of allogeneic cells and anti-hapten antibody titers were determined by a Farr technique. By a Farr technique antibody affinity was determined for sera collected 10 days after receipt of allogeneic cells. By a modified Jerne plaque technique the distribution of antibody affinities produced by guinea pig spleen cells was determined at 10 days after receipt of allogeneic cells. And, finally, the inflammatory response to an intradermal injection of croton oil was evaluated at 6 days after the receipt of allogeneic cells.

Major Findings:

In comparison to similarly primed control guinea pigs, the animals which also received allogeneic lymphoid cells were found to have:

1) an augmented delayed hypersensitivity that was carrier specific.
2) an increased production of MIF in response to antigen in vitro by peritoneal exudate cells.
3) a spontaneous rise in the anti-hapten and anti-carrier antibodies and an augmented secondary antibody response to the hapten carrier conjugate used in priming.
4) a higher affinity anti-hapten antibody at 10 days after receipt of allogeneic cells
5) an increase in the number of spleen cells producing anti-hapten antibody
6) a large population of very high affinity antibody producing spleen cells
7) an augmented inflammatory response to croton oil

Significance to Biomedical Research and the Program of the Institute:

These studies demonstrate that a mild, transient GVH reaction boosts a primed guinea pig's immune and inflammatory responses. With respect to the immune response both the effects of the cellular immune response and humoral immune response are augmented. The exact mechanisms and kinetics are, however, unknown. Recalling that strain 2 guinea pigs with L_2C leukemia have prolonged survival after receipt of allogeneic lymphoid cells, we are enticed by the possibility that one, two, or all three of the augmented immune and inflammatory functions are responsible for prolongation of survival and that the "allogeneic effect" may be useful in immunotherapy of human tumors.

Proposed Course of the Project:

The role of the inflammatory response (macrophages) in the augmentation of the effects of the cellular immune response (DH and MIF production) will be examined to further delineate the mechanism of the allogeneic effect. Then the mechanism of prolonged survival of leukemic animals given allogeneic lymphoid cells will be investigated further.

Project Subtitle V:

Project Description:

Objectives:

Several in vitro models of lymphoid cell mediated cytotoxicity have been described. In one instance lymphoid cells from sensitized animals may cause destruction of target cells. By contrast, unsensitized lymphoid cells may destroy antibody-coated target cells in the absence of exogenous complement - antibody dependent lymphoid cell mediated cytotoxicity. Our study of antibody dependent lymphoid cell mediated cytotoxicity aims to determine:

1) The identity of the effector cell.

2) The mechanism of target cell destruction including the role of complement and the nature of the effector cell surface receptors.

3) Its biological role.

Methods Employed:

The ability of lymphoid cells to effect antibody dependent cytotoxicity was evaluated by the release of ^{51}Cr from antibody sensitized burro erythrocytes. The erythrocytes were sensitized with heat inactivated guinea pig antiserum to burro erythrocytes. Spleen cells from thymus deprived mice, and spleen cell populations from which T lymphocytes had been removed by treatment with anti-θ antiserum and complement (C) were tested for their ability to release ^{51}Cr from these erythrocytes. Also the effect of pretreatment of the lymphocytes with rabbit anti-mouse K chain antibody in the presence or absence of complement was evaluated. The organ distribution of these cells was examined as well.

To determine whether B lymphocytes or other lymphoid cells such as monocytes were mediating the cytotoxicity, rosettes were formed as follows: murine bone-marrow derived (B lymphocytes) have a receptor for C_3 and can therefore form clusters (rosettes) with sheep erythrocytes that have been sensitized with antibody and complement (CRL or complement receptors lymphocytes). Cluster formation with polymorphs and macrophages can be prevented if magnesium is removed during incubation and if purified 19S anti-sheep red cell antibody is used.

These rosettes are denser than non-rosetted cells and can therefore be removed from cell populations by sedimentation. This is accomplished by centrifugation on bovine serum albumin gradients. The cytotoxic properties of the remaining cells can then be tested. Furthermore, we were able to enrich populations in CRL and test for cytotoxic activity. This was done by dissociating the sedimented rosettes and removing the sheep erythrocytes on further gradients. It was found that mouse plasma was an effective agent to dissociate these rosettes. Finally, the effect of depletion of adherent cells by rayon wool column passage or incubation on plastic surfaces was determined.

Major Findings:

Spleen cells from thymus deprived mice were fully competent to mediate destruction of antibody coated burro erythrocytes. In vitro treatment of spleen cell populations from normal mice with anti θ serum and C also did not diminish the cytotoxicity. However, pre-treatment of spleen cells in vitro with anti k serum with or without C removed cytotoxic activity from the spleen population. These studies suggested that the effector cell was a B lymphocyte. However, passage of cells through adherence columns removed most of the cytotoxic activity and furthermore, although lymph nodes contain a significant proportion of B cells the cytotoxic activity of B cells was very low as compared to spleen cells. These findings suggested that monocytes could also play a role in this type of cytotoxicity.

To resolve this problem, specific B cell rosettes were formed and populations of spleen cells were either depleted and enriched in B cells and these depleted and enriched populations were then assayed for their cytotoxic activity. In all cases the degree of cytotoxic activity of the cells followed the B cell content of the cell population. Thus, overall these findings strongly suggest that B lymphocytes or rather a subclass of B lymphocytes are involved, this subclass is as yet undefined and further studies will be needed to definitively establish the identity of the effector cell.

Our previous studies showed that lymphocytes from guinea pigs deficient in the fourth component of complement showed no diminution of cytotoxicity. We have subsequently shown that mice congenitally deficient in the fifth component of complement similarly showed no decrease in the capacity to lyse target cells. This finding rules out involvement of any known complement pathway.

Significance to Biomedical Research and the Program of the Institute:

Antibody dependent lymphoid cell mediated cytotoxicity may have considerable immunopathological significance; experimental evidence for a role in the pathogenesis of certain autoimmune diseases and graft rejection has been found.

Project Subtitle VI: Surface Immunoglobulins of Human Lymphocytes--Presence of a High Proportion of IgD-Bearing Lymphocytes

Project Description:

Objectives:

During the course of studies on the presence of immunoglobulin subclasses on lymphocytes we observed that a fluoresceinated anti-IgD antibody stained a high percentage of peripheral blood lymphocytes. Since IgD represents only a very minor subclass of human IgG in serum, this finding was quite surprising. Studies were therefore performed to confirm this observation.

Methods Employed:

Blood lymphocytes were purified by dextran sedimentation and rayon wool column filtration. Direct immunofluorescence was used to demonstrate surface immunoglobulin on lymphocytes. Thus, lymphocytes were incubated with fluorescein-labeled goat anti-human γD. This antibody was rendered monospecific by serial immunoadsorptions with 1) DEAE Fraction II (Pentex), 2) purified light chains, 3) serum from a donor with known low levels of IgG, each coupled to cyanogen bromide-activated Sepharose columns. Naturally occurring antilymphocyte serum was removed by absorption with spleen cells. Specificity was demonstrated by lack of fluorescein staining with anti-γD serum that had been adsorbed with sepharose coupled to purified γD. The morphology of fluorescence cells was determined by staining with giemsa after localization of individual fluorescent cells by microscope stage coordinates. The total number of immunoglobulin bearing lymphocytes was determined by immunofluorescent staining with anti-Fab antiserum.

Major Findings:

A remarkably high percentage of lymphocytes showed a bright speckled pattern of immunofluorescence with anti-IgD. Thus about 20% of immunoglobulin bearing cells (mean of 10 volunteers). stained in this manner; the normal mean serum level of γD is only 30 μg/ml as compared to a level of 12.5 mg/ml for γG. The number of γD bearing cells was not found to correlate with serum IgD levels in individual donors. The cells staining with anti-IgD had the morphological features of small lymphocytes. No IgD positive staining cells were found in the thymus gland.

Significance to Biomedical Research and the Program of the Institute:

This discovery may have considerable potential significance. The unexpectedly large number of γD bearing peripheral blood lymphocytes point towards a functional role for these cells and for γD immunoglobulin.

Proposed Course of Project:

Presence of IgD bearing lymphocytes in other lymphoid organs will be investigated. Studies to determine if the cell bearing IgD is producing this immunoglobulin will be carried out. Relative percentages of cells bearing other immunoglobulin classes will also be determined.

PUBLICATIONS:

Shevach, E.M., Ellman, L., Davie, J.M. and Green, I.: L_2C guinea pig lymphatic leukemia: A "B" cell leukemia. Blood 39: 1, 1972.

Katz, D.H., Ellman, L., Paul, W.E., Green, I. and Benacerraf, B.: Resistance of guinea pigs to leukemia following transfer of immunocompetent allogeneic lymphoid cells[1]. Cancer Research 32: 133, 1972.

Ellman, L., Katz, D.H., Green, I., Paul, W.E. and Benacerraf, B.: Mechanisms involved in the anti-leukemic effect of immunocompetent allogeneic lymphoid cell transfer[1]. Cancer Research 32: 141, 1972.

Shevach, E., Herberman, F., Lieberman, R., Frank, M.M. and Green, I.: Receptors for immunoglobulin and complement on mouse leukemias and lymphomas. J. Immunol. 108: 325, 1972.

Van Boxel, J., Stobo, J., Paul, W.E. and Green, I.: Antibody dependent lymphoid cell mediated cytotoxicity. Lack of requirement for T lymphocytes. Science 175: 194, 1972.

Shevach, E., Green, I., Ellman, L. and Maillard, J.: Heterologous antiserum to thymus derived cells in the guinea pig. Nature 235: 19, 1972.

Ellman, L., Inman, J.K. and Green, I.: Strain difference in the immune response to hydralazine in inbred guinea pigs. Clinical & Exp. Immunol. 9: 927, 1971.

Van Boxel, J., Steinberg, A. and Green, I.: A study of the antibody mediated immune responses to ribonucleic acids in guinea pigs. J. Immunology. 108: 446, 1972.

Shevach, E.M., Stobo, J.D. and Green, I.: Immunoglobulin and theta bearing murine leukemias and lymphomas. J. Immunol. In press.

Shevach, E.M., Herberman, R., Frank, M.M. and Green, I.: Receptors for complement and immunoglobulin on human leukemic cells and human lymphoblastoid cell lines. J. Clin. Investigation. In press.

Elfenbein, G.J., Shevach, E.M. and Green, I.: Demonstration of thymus derived cell surface antigens on various guinea pig lymphoid cell populations by a microimmunoadherence technique. Immunology. In press.

Serial No. NIAID - 144
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The Cellular Basis for Antibody Formation

Previous Serial No.: 144

Principal Investigator: Dr. Bruce Merchant

Other Investigators: Project Subtitle I: Dr. John Inman
Dr. Gerald Vurek, LTD, NHI
Dr. Edgar E. Hanna, LMB, NICHHD
Project Subtitle II: Dr. John Inman
Dr. Michael Potter, LBGY, NCI
Dr. Rose Mage
Project Subtitle III: Dr. Latham Claflin

Cooperating Units: Dr. Joseph Ingraham, Dept. of Microbiology, University of Indiana Medical Center, Indianapolis, Indiana.
Dr. P. Liacopoulos, Institut D'Immuno-Biologie, Hopital Broussais, Paris XIV^{e}, France.
Dr. Zacharie Brahmi, Pasteur Institute, Paris, France
Dr. Thomás Hraba, Czechoslovak Academy of Sciences, Institute of Experimental Biology and Genetics, Na Cvicisti 2, Prague 2, Czechoslovakia.

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2 10/12		2/12	3
Professional	2		2/12	2-2/12
Other	10/12			10/12

Project Subtitle I: Cellular participation in the immune response to chemically defined antigens.

Project Description:

Objectives: To determine whether precursor cell activation is best accomplished by standard conventional haptens or by extended and

enlarged forms of the same haptens coupled to the same immunizing carrier. Also, to ascertain whether the selective processes operative during long term immunization favor preferential reactivity toward the actual immunizing determinant or toward some extended form of this immunizing determinant.

Methods Employed:

Rabbits are immunized with equal amounts of antigen which bear either classical azohaptens (e.g., para-azobenzenearsonate, para-azobenzenesulfonate, etc.) or the same azohaptens enlarged and extended by use of a tripeptide "spacer" or stal of known sequence, the use of which causes the whole haptenic grouping to approximate the estimated size of the average antibody combining site. Lymphoid cell suspensions from immunized rabbits are assayed for their contained numbers of hapten specific plaque forming cells (PFC) directed either against the immunizing hapten or against any of 8 to 12 structural analogues. The determined activities constitute a profile of antibody forming cell commitment, both with respect to specificity and with respect to immunoglobulin class. The specificity-class profile of any given immune cell suspension is characteristic of it and may be used to identify it.

Two types of profile determinations are normally performed for each major class (IgM or IgG) of PFC. One standard azohapten profile is run with each lymphoid cell suspension. Another similar profile is determined for the extended or enlarged forms of the corresponding azohaptens. For simplicity and convenience we will refer to standard azohaptens as small and to the enlarged or extended forms of the same azohaptens as large.

The main feature of the large hapten profile is that it employs tripeptide "spacers" subjacent both to the immunizing azohapten and to the assay azohapten (and its various analogues) in the dozen different indicator erythrocyte systems comprising our large hapten assay profile. These dozen unique azohapten tripeptide molecules of known structure, uniformity, and sequence were planned and synthesized by Dr. John Inman. Our main immunizing haptens have been para-azobenzenearsonate, para-azobenzenesulfonate, and para-azophenyl, each separately and covalently linked to acetyl-tyrosyl-glycyl glycine which is then coupled by an azide linkage to the epsilon amino group of lysine on either duck erythrocyte membranes (our immunogen) or on the surface of sheep erythrocytes (our indicator system). Three of the large haptens in our series are coupled similarly via a ß-alanyl-glycyl-glycyl tripeptide.

Definitions:

Our findings have necessitated the introduction of certain new terms and explicit definitions of others. We define antibodies as all intact functional new immunoglobulin molecules elicited by an antigenic stimulus. Antibodies may be either specific of quasispecific for the immunizing antigen (or hapten). Antibodies reacting more strongly with the immunizing hapten than with any other test hapten are termed specific antibodies, and they are identical with traditional antibodies. Other new immunoglobulin molecules

more strongly reactive with a structural analogue than with the immunizing hapten are termed quasispecific antibodies, or "quasibodies" for short.

Major Findings:

The present series of investigations permitted a comparison of large vs. small azohaptens with regard both to precursor cell activation and to assay efficiency. Initial experiments were performed to demonstrate that both large and small azohapten profile assay systems were operating at optimal sensitivity. Thereafter, it became abundantly clear that the large azohaptens were consistently and profoundly the more potent immunogens. Other important findings fall generally into three categories.

A. Cell populations elicited by immunization with a large azohapten.

1. Lymphoid cell suspensions from large hapten immunized rabbits regularly demonstrate about 10 times more specific plaque forming cells than are regularly seen for the corresponding specific small hapten.

2. Large hapten immunization regularly elicits a broad large hapten analogue profile but elicits only a very narrow small hapten analogue response. Sometimes, only the specific small hapten response can be detected.

3. Quasibody secreting cells reactive with large hapten analogues are many times more numerous than plaque forming cells releasing molecules specifically reactive with the small form of the immunizing large hapten.

B. Cell populations elicited by immunization with a small azohapten.

1. Regardless of the specific small hapten PFC activity against the immunizing small hapten, plaque forming cells detected with the corresponding large hapten always ranged from 2 to 12 times more numerous. This was extraordinary since precursor cells had never officially "seen" the large form of the immunizing hapten.

2. This finding was true both for IgM and IgG plaque forming cells.

3. This finding was also true both of early and of late immune responses for periods up to 2-1/2 years.

4. When quasibody releasing (i.e., analogue reactive) PFC were seen in the small hapten profile, other PFC were always more numerous for the corresponding specificities in the large hapten profile.

5. In fact, quasibody releasing large hapten reactive plaque forming cells frequently outnumbered specific antibody releasing small hapten reactive plaque forming cells, especially in azobenzene sulfonate immunized rabbits.

C. Unimmunized rabbits.

1. Spontaneous "background" IgM PFC activities regularly range from 5 to 10 times higher for any given large hapten specificity than for the corresponding small hapten specificity.

Significance to Biomedical Research and the Program of the Institute:

These results reveal the potent immunogenicity of the large azohaptens and they reaffirm the remarkable sensitivity of the large hapten assay systems. They also raise questions about the sensitivity of many plaque forming cell studies performed with conventional haptens of usual size (i.e., with dimensions sufficient to fill less than 1/2 of the estimated average antibody combining site). The present investigations also suggest that encounter with a small hapten may serve to activate certain precursor cells which are commited to the release of molecules having combining sites far more reactive in hemolytic reactions with chemically identifiable alternate structures.

The preferred alternate structures are extended forms of the same determinant which possess the same immunodominant distal or presenting azohapten tip. Presumably, this reflects, at least in part, an increased accessibility to reaction offered by the enlarged or extended form of the azohapten. However, the contribution of the subjacent "spacer" peptides at least to critical effector interactions is often sufficient to more than offset marked variations in the azohapten terminus. Such approaches permit us to determine quite precisely the chemical and structural specifications for B cell activation and for the triggering of hemolytic effector mechanisms. Such knowledge may prove most beneficial to the ultimate specific manipulation of adverse immune responses, especially for those responsible for humoral type autoimmune disease.

Proposed Course of Project:

The program-frozen cells from these animals will be used for a variety of affinity and avidity studies. The generality of these findings is being tested by extending them to inbred mice. Whether certain animals, heretofore considered unresponsive, may in fact harbor quasibody secreting cells is also under active investigation

Project Subtitle II: Genetic Contributions to Specific and Quasispecific Antibody Production in Native Germ-Free and Conventional and in Immunized Mice.

Project Description:

Objectives:

To determine baseline vs. active immune status of inbred and random bred mice with respect to large hapten profile plaque activities.

Methods Employed:

The basic experimental and assay methods are essentially the same as those outlined under Subtitle I, above, except that for most studies only the large hapten profile determinations have been performed.

Major Findings:

In our experience, as in most reports recorded in the literature, the usual level of spontaneous IgM antibody activity against sheep erythrocytes in most laboratory species, is around one plaque forming cell per 10^6 nucleated spleen cells. In all probability, this represents the sum total of spontaneous background PFC populations reactive with each of a dozen or more distinct antigens represented on sheep erythrocyte surfaces. Previous studies from other laboratories have clearly indicated that the appearance of the "spontaneous" background anti-sheep red cell activities depend upon postnatal exposure to environmental antigens.

Since none of the 14 synthetic chemical azohaptens employed in our large hapten profile are known to exist in nature, it might be expected that spontaneous background PFC activity to most of these test haptens would be close to zero, or at least would not exceed about 0.1 plaque forming cells per 10^6 nucleated lymphoid cells.

To regularly observe spontaneous background values for large hapten specific PFC averaging around 100 fold higher was unexpected, to say the least. In conventional inbred and random bred mice, these spontaneous background hapten specific PFC activities commonly range from 2 (for the lowest specificity) to around 30 (for the highest) plaque forming cells per 10^6 nucleated spleen cells. Spontaneous background activity against corresponding small azohaptens averages only around one PFC per 10^6 spleen cells. For a representative mouse strain (e.g., BALB/c mice), the average large hapten spontaneous background activity for each of the 14 test haptens is about 10 PFC per 10^6 nucleated spleen cell.

The large hapten specific PFC values average approximately 5 per 10^6 per specificity for C57 Bl/6N and CBA/N mice, approximately 10 per 10^6 for outbred general purpose albino mice, and nearly 20 per 10^6 for New Zealand Black (NZB) mice. The elevated spontaneous background PFC activities in NZB mice is intriguing. It may relate in some way to their propensity to develop humoral autoimmune diseases. It is of interest that for NZB mice an apparent inverse relationship exists between natural and acquired immunity to our large azohaptens. Although they have the highest average spontaneous IgM activities of any mouse strain yet studied, they also manifest, after active immunization, the feeblest and most diverse IgM responses yet observed. In one set of experiments, an immunization schedule which caused a 50 fold rise in specific large hapten reactive PFC in BALB/c mice, resulted in New Zealand Black mice in only a 4 fold increase for the corresponding profile specificities.

Spontaneous IgM PFC activities are profoundly elevated in spleens of late gravid and recent postpartum female mice. Values as high as 700 PFC per 10^6 have been recorded. These "spontaneous" activities are double or triple the usual peak active immune response values recorded in the literature for mice following in vivo immunization. Such elevated spontaneous background IgM PFC do not appear to be related to fetal genotype since comparable elevations are seen in gravid females carrying either semi-allogeneic or syngeneic fetuses.

Absolute spontaneous IgM anti-large hapten PFC activities in germ-free mice are normally even 2 to 3 fold higher per 10^6 nucleated spleen cells than for conventionally reared mice of the same age and strain. Since spleens of germ-free mice are small this amounts, however, to only modest increases in large hapten specific PFC activity per spleen.

Finally, the configurations of large hapten specific IgM PFC profiles in conventional BALB/c mice are very similar from one individual mouse to the next. In individual germ-free BALB/c mice, these profiles are practically identical. In contrast, random bred or general purpose albino mice having similar average IgM PFC activity levels show wide profile fluctuations from one individual to the next. This is true both of conventional and of germ-free general purpose mice. Among conventional inbred mice characteristic and reproducible profile differences exist from strain to strain, such that each strain thus far studied can be identified by its spontaneous background IgM anti-large hapten profile.

Significance to Biomedical Research and the Program of the Institute:

As in most investigations of biologic phenomena, precise assessments of the relative contribution of inheritance and of environment are exceedingly difficult. Often, the final evaluation involves judgments of degree.

It could easily be argued that all "spontaneous" background IgM PFC activity in germ-free mice arises as a result of encounter with antigenic materials ingested in a sterilized diet. Although this is a valid possibility, it fails to readily explain the wide profile variations seen between individual random bred germ-free mice, all of which ingest the same diet. Stimulation by diet-derived antigen might also be expected to be far more intense in conventional mice which consume unsterilized diets and which regularly indulge in coprophagy. Nevertheless, absolute spontaneous background IgM PFC activities regularly range somewhat lower in conventional than in germ-free mice of the same age, sex and strain. It also appears unlikely that dietary antigen could readily explain the profound elevations in spontaneous background PFC activities observed in gravid and postpartum female mice.

Since characteristic differences exist between the spontaneous IgM large hapten profile from one inbred strain to another and since within a given strain these same 14 parameter profiles are essentially identical from one individual to the next, the findings speak in favor of rather strict genetic control over spontaneous IgM antibody specificity and synthesis. Such findings by no means rule out possible somatic contributions to antibody specificity. They merely serve to emphasize the role of possible genetic contributions. In this respect, they complement findings reported under Subtitle III below, and findings included in last year's report. On the other hand, such findings do raise the possibility that IgM antibody formation is under exclusive germ line control. If this is so, certain autoimmune diseases may ultimately be susceptible to Eugenic control.

Proposed Course of Project:

Other species and other mouse strains are being studied to ascertain whether the above findings constitute general biologic phenomena. Special emphasis is being placed upon studies with germ-free and conventional NZB mice and with their semi-allogeneic offspring. The ontogeny of spontaneous IgM PFC profile development is also under active investigation.

Project Subtitle III: Cellular Basis for Maturation of the Immune Response in Mice.

Project Description:

Objectives:

To characterize at the cellular level the nature of events leading to time dependent increases in the binding affinity of serum antibodies.

Methods Employed:

Mice were immunized with an enlarged hapten, dinitrophenyl-β-alanyl-glycyl-glycine (Dnp AGG) coupled to a protein carrier. At intervals after immunization, measurements of the binding affinities of hapten specific antibodies present in the serum and secreted by antibody-forming cells were performed. A modification of the Farr technique was used to assess the antigen binding properties of serum anti-Dnp AGG antibodies. Inhibition of plaque forming cells (PFC) by free hapten was used to determine the relative ligand binding of anti Dnp AGG antibodies secreted by PFC populations.

Major Findings:

1. A time dependent maturation of the anti-Dnp AGG immune response occurred in BALB/c mice immunized with Dnp AGG_{20}-KLH. This process was recognized at the humoral level by an increase with time in the average association constant of serum antibodies and at the cellular level by a decrease with time in the concentration of free hapten required to obtain 50% reduction in PFC.

2. Maturation of the immune response could be demonstrated for both IgM and IgG PFC. Maturation for IgM PFC was virtually complete 7 to 9 days after immunization, whereas maturation of IgG/PFC required at least 30 days. Attempts to "extend" the maturation process beyond that seen in a late primary (day 30) response did not succeed. Methods used for this purpose involved A) extended periods of immunization; B) varying the dose of antigen used for immunization; C) preimmunization of mice with carrier only; D) boosting with hapten on a heterologous carrier.

3. The manner by which the slopes of the IgM and IgG PFC inhibition curves changed, suggested that maturation occurred as the result of a simple cell selection process, and it appeared to involve primarily precursor cells existing prior to administration of antigen.

Significance to Biomedical Research and the Program of the Institute:

The differentiation of the immune system (particularly of those lymphocytes responsible for antibody production) is a fundamental immunologic process. Elucidation of the nature of this process would be of obvious immunologic significance.

Proposed Course of Project:

Present investigations will be continued with particular emphasis placed on characterizing the maturation process during ontogeny.

Publications:

Hanna, E.E. and Merchant, B.: Secretion of monospecific anti-hapten IgG antibodies by rabbit immunocytes. J. Immunol. 107: 172-184, 1971.

Claflin, L. and Merchant, B.: Restricted maturation of antibody binding characteristics for hapten-specific IgM-plaque-forming cells in mice. Cell. Immunol. In press.

Merchant, B. and Liacopoulos, P.: Paradoxical cellular and serologic responses following antigenic competition. Int. Arch. of All. and Appl. Immunol. 42: 516-532, 1972.

Serial No. NIAID - 145
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Cellular and Molecular Structure Studies of Antibody Specificity and Function

Previous Serial No.: 145

Principal Investigator: Dr. John K. Inman

Other Investigators: Project Subtitle I: Dr. Bruce Merchant
Dr. Latham Claflin
Project Subtitle II: Dr. Ettore Appella (NCI)
Dr. Rose G. Mage

Cooperating Units: Project Subtitle II: Dr. Ettore Appella, Laboratory of Cell Biology, National Cancer Institute
Dr. Allan Grossberg, Roswell Park Memorial Institute, Buffalo, N.Y.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	1-10/12			
Professional	1			
Other	10/12			

Project Subtitle I: Cellular Studies on the Development of Humoral Immune Responses Directed Toward Large, Synthetic Haptens

Project Description:

Objectives:

To apply methods of chemical design and synthesis toward optimization and extension of the hemolytic plaque assay for studying populations of specific antibody secreting cells. To direct these studies toward questions concerning the nature of: 1) the structural genes coding for antibodies, 2) precursor cells for specific antibodies, 3) the specificity of precursor cell activation, 4) antibody specificity and avidity during maturation of primary and secondary immune responses, 5) antibody heterogeneity and,

6) multisite interactions at cell surfaces.

Methods Employed:

A group of 14 compounds were synthesized according to structural features designed especially for hapten specific hemolytic plaque studies. The compounds provide a variety of terminal haptenic structures connected by common linkage units to a functional group that can be activated to form covalent bonds. The bonds are formed under mild conditions in aqueous media with available amino groups on immunogen carriers, test red cell surfaces or soluble proteins. The haptenic structures thus introduced are large enough to fill most antibody combining sites and thereby minimize the contribution of unknown carrier substructures to antibody specificity.

The compounds, which are derivatives of several tripeptides, have been characterized by functional group tests, elemental analyses, amino acid analysis, and by their absorption spectra. Each compound possesses a tertiary butyloxycarbonyl hydrazide group that can be converted to an active acyl azide by a low temperature reaction in small volumes of dimethylformamide. All 14 compounds are routinely converted simultaneously and added to separate suspensions of sheep erythrocytes for preparing specifically sensitized test cells.

In collaboration with Dr. Bruce Merchant and Latham Claflin hemolytic plaque studies were carried out with a variety of experimental designs. Lymphoid cells were obtained from normal and immunized rabbits, mice and guinea pigs and mouse myeloma tumors. Plaques were studied against singly and doubly coupled test cells, with and without incorporation of soluble haptenic inhibitors in the assay medium. Soluble inhibitors were varied in concentration and consisted of the unconjugated compound (monovalent inhibitors) and lightly or highly conjugated protein (polyvalent inhibitors).

Immunogens were prepared by covalently coupling the large haptens to Peking duck erythrocyte stromata in dimethylformamide, or to soluble proteins such as bovine serum albumin and keyhole limpet hemocyanin in aqueous media.

The absolute number of hapten molecules coupled to test erythrocytes under optimal conditions (concentration, time, pH) for plaque assays was estimated. One of the haptens was radioiodinated on its terminal tyrosine side chain with ^{125}ICl prepared by exchange of $^{125}I^-$ with unlabeled ICl. The amount coupled was determined from the calculated specific activity and measured gamma counts. Amounts of hapten coupled to cells having less than the optimal degree of conjugation were also determined.

Methods of synthesis of several of the hapten compounds were improved by employing Sephadex ion-exchangers in place of cellulose derivatives, and by use of special extraction procedures.

Major Findings:

The method of coupling large haptens to test red cells developed in this study has consistently led to stable preparations giving reproducible plaque counts from batch to batch for aliquots of the same frozen cell suspension. The resulting sensitized red cells are usable for three to four weeks as compared with several days to a week for cells prepared by other methods of conjugation. Plaquing efficiency was determined for tumor cells secreting a protein reactive toward dinitrophenyl (DNP) groups (murine myeloma MOPC 315) using test cells carrying the DNP-large hapten. Essentially the theoretical limit (about 90% of all tumor cells form plaques as determined by Dr. Claflin) was observed. Evidence for the high sensitivity of the hapten specific plaque technique as now employed in studies of various normal lymphoid tissue is reprted in the corresponding report by Dr. Merchant.

Optimum conditions for preparing hemolytic plaque test cells have been carefully checked. The binding of ^{125}I-labeled hapten to sheep red cells increases as the concentration of hapten azide is increased in the reaction mixture. An optimum degree of coupling with respect to plaquing efficiency occurs when about 250,000 molecules are bound to each red cell. Above, as well as below, this level plaque counts decrease. In as much as the single-hit hypothesis of complement dependent, specific hemolysis appears valid, the above optimum conjugation figure strongly implicates the combined requirement for multisite interaction between antibody and red cell membrane and a fortuitously correct spacing (and orientation) of a few haptenic groups somewhere on the membrane.

Data continues to accumulate which demonstrate that cells secreting antibodies to alternate haptenic structures (including both close and not-so-close analogs) frequently do not form plaques with test cells possessing the immunizing hapten; and, vice versa, those cells plaquing against the immunizing antigen usually do not plaque against an alternate structure. Thus, the selective character of the antibody specific interaction in complement dependent hemolysis allows the enumeration of many separate groups of antibody secreting cells that arise in response to a single immunogen. Plaque assays carried out with soluble inhibitors at various concentrations allow an assessment of relative binding avidity of antibodies from the different groups. The use of monovalent inhibitors permits an ordering of groups according to single combining site affinities. Such studies show that, for the most part, affinities are highest for all groups (as distinguished by different test red cell haptens) when the soluble inhibitor is homologous with the immunogen. That is, the combining sites individually combine best with the immunizing structure*. When polyvalent

*We have some evidence that in the earliest states of primary immune response there are some antibodies whose combining sites individually combine better with an alternate, monovalent hapten and are thus truly "degenerate" or "heteroclitic" antibodies.

inhibitors are employed, much the same result is obtained only with the lightly conjugated proteins (about 5 groups per BSA molecule). With highly coupled protein (> 20 groups per molecule), the inhibitor homologous with the test red cell hapten shows higher avidity (inhibits at lower concentration) than the inhibitor which is homologous with the immunogen. Thus, the interactions of antibodies with the highly substituted inhibitors imitate with regard to specificity those occurring at the red cell surface.

The hypothesis used to explain the selective specificity profiles is based upon the assumption of a multisite interaction and the importance of the relative orientation of antibody combining sites and the presenting haptenic groups on the red cell surface. Alternate haptens with lower single-site affinities may be able to combine simultaneously with two or more sites more advantageously than the homologous hapten because of orientational preference or flexibility and thereby to give rise to a more highly avid interaction (or at least one more productive in exposing a complement fixing site). In this regard, high affinity sites may impose orientational demands which are too strict for effective multisite attachment.

It would appear that the specificity of the initial activation of precursor cells in a primary immunization is appropriate in terms of the receptors acting like single, independent combining sites similar to those of antibody eventually secreted. But we have evidence (Dr. Merchant) that maturation of the immune response with respect to specificity of the IgG secreting cells(and not IgM cells) follows the selective logic of the hemolytic plaque assay; that is, clonal competition, more than the initial stimulation, involves the advantages of simultaneous multisite interactions at cell surfaces.

A number of other findings, especially those with germ-free and inbred mice, resulting from the specific PFC studies are discussed in Dr. Merchant's report. The use of the enlarged haptens has made it possible to achieve good anti-hapten immune responses in mice and to detect the corresponding plaque forming cells efficiently whereas poor results were usually encountered with conventional small haptens.

Significance to Biomedical Research and the Program of the Institute:

These studies bear upon fundamental questions concerning the origin, nature, and development of immunological competence and specificity. Some interesting ideas are taking shape which relate specificity to possibly important geometrical factors that operate when antibodies or antibody-like receptors interact with repetitive antigenic determinants on cell or antigen surfaces. These studies appear also to bear on genetic factors which determine the expression of specific antibodies.

Proposed Course of Project:

The hapten-specific hemolytic plaque studies are to be pursued vigorously. The effect of various immunizing carriers will be explored. Continuing emphasis will be placed on studies with inbred animals and the roles played by genetic versus random or environmental factors in determining the character of immune responses.

Project Subtitle II: Structural Studies on Immunoglobulins and Antibodies

Project Description:

Objectives:

To develop new methodology in the determination of amino acid sequences in proteins and immunoglobulins, and to combine these findings with conventional techniques for determining primary structures of antibody molecules. To elucidate the nature and operation of the structural genes for antibodies, and to explore some questions concerning the structure and manner of functioning of the antibody combining site.

Methods Employed:

The reagent for initiation Edman degradations, 4-sulfophenylisothiocyanate, suggested by Braunitzer et al., was synthesized by the reaction between sulfanilic acid and thiophosgene. The method was used for preparing this compound with a ^{14}C label after first preparing ^{14}C-sulfanilic acid from radiolabeled aniline by a method also developed in this laboratory. A convenient manual coupling procedure for initiating sequential degradations of peptides was worked out and used successfully in the automated sequenator program in collaboration with Dr. Appella.

Improved methods were worked out for preparing N-thiobenzoylamino acid amides, esters and hydrazides for studies of a new degradation technique for sequencing amino acids. New analogues of the NIP-(3-nitro-4-hydroxy-5-iodophenacetyl-) hapten were prepared by conventional synthetic steps. A modified Farr assay for antibody binding affinity was worked out according to the approach of Dr. Walter Hughes (Tufts Univ., personal communication) where ammonium sulfate precipitated antibody-hapten complexes are counted for radioactivity on micro-filters to determine percent bound hapten.

In collaboration with Dr. Rose Mage, attempts are being made to hyperimmunize rabbits with protein conjugates of some of the large tripeptidyl haptens employed in the hemolytic plaque studies. The sera are being examined by gel-slab isoelectric focusing (IEF) followed by a staining procedure for specific antibody developed in this laboratory: the proteins in the IEF pattern are fixed with conc. Na_2SO_4 and reacted with a conjugate of horseradish peroxidase and hapten. Excess conjugate is removed, and bound hapten-enzyme complex is visualized by adding a chromogenic peroxidase substrate.

The peroxidase conjugates were prepared with hapten azides after first introducing reactive, spaced-out amino groups into the enzyme (its own amino groups being inadequately reactive). The spacer diamine was bis-glycinamidoethane, a highly water-soluble compound possessing terminal α-amino groups. A convenient, inexpensive method for its synthesis was worked out. The compound was used also as a spacer on an immunoadsorbent used for the purification of the NIP-binding murine myeloma protein S-183.

Major Findings:

In collaboration with Dr. Appella and Mrs. Hannon (NCI), a study was made to compare yields of phenylthiohydantoin amino acids at successive stages of Edman degradations which were initiated in the first step by 4-sulfophenylisothiocyanate and, alternatively, by the usual reagent, phenylisothiocyanate. This method, suggested by Braunitzer and coworkers, was adapted to a simple, manual, initial coupling step and subsequent automatic operation in the Beckman Sequencer programmed for volatile buffers. Tryptic peptides, terminating in lysine or aminoethylcysteine, which are quickly lost in extraction steps in the usual procedure, were retained in the modified method sufficiently well to allow completion of sequencing in most cases. These results have been published recently (see Publications).

Work is in progress to modify and adapt the Barrett-Previero sequential degradation of peptides to schemes for the identification and quantitation of residues by liquid chromatography. Simple methods have been worked out for converting intermediate thiazolinones to stable thioacylamino acid amides and hydrazides which can be separated readily by thin layer chromatography.

The method of staining specific antibodies after IEF electrophoresis or gel diffusion runs by the use of peroxidase--hapten conjugates has proved to be a useful tool in evaluating the heterogeneity of specific antibody. The procedure is simpler to carry out than the radioautographic methods, and results are obtained sooner.

A murine myeloma protein, S-183, reported to bind the NIP hapten was purified and found to bind radioiodinated NIP compounds and homologs with association constants ranging from 10^3 to 10^4 M^{-1}. These values were obtained in this laboratory and by Dr. Allan Grossberg at Roswell Park Memorial Institute. The binding strengths are considered too low for active site labeling studies. Other haptens or analogs may be tested.

Significance to Biomedical Research and the Program of the Institute:

A clear understanding of the number and mode of expression of structural genes for immunoglobulins will be possible only after careful examination of much more amino acid sequence data than is presently available. The relationship of structural genes and amino acid sequences to antibody specificity and function will best be elucidated by molecular structure studies of antibodies of known specificity and limited heterogeneity, preferably those raised in response to a deliberate immunization. Basic knowledge of this sort will

help toward understanding and eventually controlling pathological events involving specific antibodies.

Proposed Course of Project:

Efforts will be continued toward improving sequence methodology though 1) redesigning the chemistry of sequential degradation, 2) finding means of rapid quantitation of degradation products through advanced liquid chromatographic techniques, 3) employing the latter techniques toward peptide separations (including affinity chromatography) and 4) adapting these improvements to automated sequencing.

PUBLICATIONS:

Martin, W.J., Wunderlich, J.R., Fletcher, F. and Inman, J.K.: Enhanced immunogenicity of chemically coated syngeneic tumor cells. Proc. Nat. Acad. Sci. 68: 469-472, 1971.

Rose, J.A., Maizel, J.V., Jr., Inman, J.K. and Shatkin, A.J.: Structural studies of adenovirus-associated viruses. J. Virology 8: 766-770, 1971.

Inman, J.K., Hannon, J.E. and Appella, E.: Demonstration of a simple method for reducing losses of tryptic peptides during automated sequencing. Biochem. Biophys. Res. Commun. 46: 2075-2081, 1972.

Rouques, R., Inman, J.K. and Merchant, B.: Detection of cells secreting antibodies more reactive with an alternate structure than with the immunizing hapten. Int. Arch. Allergy & Appl. Immunol. In press, 1972.

Serial No. NIAID - 146
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Chemical, Genetic and Immunochemical Studies of Immunoglobulins: Use of Genetic Markers on Immunoglobulins to Study Mechanisms and Control of Gene Expression, Genetics of Antibodies and the Immune Response

Previous Serial Number: 146

Principal Investigators: Rose G. Mage, Ph.D. and Glendowlyn O. Young-Cooper, M.S.

Other Investigators:

Dr. M. Carta	LI-NIAID
Dr. M. Harrison	LI-NIAID
Dr. J.K. Inman	LI-NIAID
Dr. E. Appella	LCB-NCI
Dr. J. Pincus	LMI-NIDR
Dr. H. Reynolds	LCI-NIAID

Cooperating Units: Dr. S. Dray, University of Illinois Medical Center Chicago, Illinois
Dr. S. Tosi, Laboratorio di Biologia Cellulare del C.N.R., Rome Italy
Dr. G. Siskind, Cornell University Medical College, New York, N.Y.

In addition, this year the following investigators have used rabbits of defined allotype which we have supplied and/or have received specific reagents which we have prepared: Dr. S. Beiser, N.Y., N.Y., Dr. J. Cebra, Baltimore, Md., Dr. S. Dubiski, Toronto, Canada, Dr. J. Engelstein, LCB-NCI, Dr. I. Green, LI-NIAID, Dr. J. Marquardt, CB-NEI, Dr. W. Paul, LI-NIAID, Dr. S. Pestka, Nutley, N.J., Dr. A. Rosenthal, LCI-NIAID, Dr. J. Stobo, LI-NIAID, Dr. B. Wolf, Philadelphia, Pa., Dr. J. Young, Berkeley, California, Dr. S. Zappacosta, Naples, Italy. We have also typed samples for numerous investigators.

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
TOTAL	4 6/12			4 6/12
PROFESSIONAL	3 6/12			3 6/12
OTHER	1			1

Project Description:

Objectives:

To define by combined immunological and chemical approaches, the chemical nature of structural differences between genetically controlled polymorphic forms of immunoglobulins and between different classes, subclasses, types and subtypes of their polypeptide chains. To utilize this information about the location and chemical nature of the markers on various chains in performing and analyzing breeding studies. To utilize reagents specific for the various chains and markers in affecting the phenotypic expression of alleles or chain types, studies of membrane associated immunoglobulins, and cell transfer studies. The overall objective is to understand the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses.

Methods Employed:

Rabbits of known allotype are bred: to produce homozygous rabbits of all combinations at three or more genetic loci; to produce heterozygotes for use in specific experiments and as F1 progeny in linkage studies; to produce F2 and backcross progeny in linkage studies; to produce allotype-suppressed rabbits; and to study the genetics of the immune responses to pneumococcal vaccines, DNP-protein conjugates, and other antigens.

Sera of rabbits from the above categories are collected and typed by gel diffusion and hemagglutination inhibition techniques for allotypes. The sera are used as sources of IgG and light and heavy polypeptide chains for chemical sequence studies and for immunizations of rabbits and other animals.

Rabbits of defined allotype are immunized with IgG, light chains or other antigens such as lysozyme, pneumococcal vaccines, hapten-protein conjugates and (for idiotype-studies) purified antibodies or pneumococci coated with anti-capsular antibodies. Antisera are tested by qualitative and quantitative immunologic and electrophoretic techniques.

Allotypes, types, subtypes and idiotypes are quantitated by: 1) radial immunodiffusion, 2) hemagglutination inhibition, 3) quantitative precipitation of purified ^{125}I-labeled proteins with specific antisera, 4) binding of labeled proteins to cross-linked antisera (immunoabsorbents), 5) inhibition of binding of labeled antigens to cross-linked antisera. Immunoabsorbents are also prepared and used to purify antibodies and antigens of known specificity.

Newborn rabbits which are offspring of: 1) normal mothers, 2) mothers immunized against a paternal allotype, 3) mothers immunized against an allotype carried in the genome of implanted zygotes, 4) allotype-suppressed mothers, are injected with anti-allotype antisera in order to suppress expression of allotypes or chain types. In studies of the mechanism of chronic allotype suppression, cells and/or plasma from allotype suppressed and control rabbits are transferred to newborn rabbits. The cell donors are allotype-suppressed fathers or full-siblings of the recipients. Cells from normal and allotype-suppressed homozygous and heterozygous rabbits are studied using ^{125}I-labeled and fluorescein-conjugated anti-allotype reagents. In addition, the cells are cultured in vitro and assayed for stimulation by a variety of materials such as allotype bearing IgG, anti-allotype antisera, and other cells.

Purified light and heavy chains or fragments of these are obtained from IgG of normal sera or specifically purified antibodies of known allotype as well as from sera of allotype-suppressed rabbits. These are studied using a large variety of methods for sequence determination including automated analysis with a Beckman sequencer.

Major Findings:

Anti-A14 and anti-A15 antisera are specific for allotypic markers on the constant regions of rabbit γG heavy chains and bind only γG while anti-a-locus antisera bind both IgG and IgM. Not only are the a1 and a2 specificities present on both IgG and IgM, but they appear to exhibit similar relative phenotypic expression under conditions of normal or altered suppressed expression. These data support concepts derived from sequence analysis that IgG and IgM share the same set of variable region genes. (Landucci Tosi, Mage and Lawton, 1972).

The A15 allotype was shown to be present on IgG from an allotype-suppressed a2 homozygote which lacked a2 and expressed a-negative allotypes (other V_H genes?). It appears therefore that a-negative specificities such as A31 are controlled at the same complex locus or chromosomal region as the other segregant groups of heavy chain determinants. (Landucci Tosi, Mage, Gilman-Sachs, Dray and Knight, 1972).

The structural basis for the A14 and A15 allotypic specificities was

investigated with Fc-fragments prepared from homozygous rabbits. The only tryptic peptide of the Fc-fragment for which the two allotypes were not identical was T19 which carried a replacement of threonine for alanine at a position homologous to residue 309 of a fully sequenced human γ1 heavy chain, Eu. It was concluded that the presence of threonine at this position correlates with the serologically detectable A14 allotype while alanine correlates with A15 (Appella, Chersi, Mage and Dubiski, 1971).

Since a recombinant rabbit which appears to have received V_H - a2 and C_H A14 from different chromosomes of its A1,14/A2,15 sire, was observed in our breeding studies, we have continued informative matings in the search for additional examples of recombination between V and C genes. We have now observed 299 informative offspring and no additional recombination has been observed. However, another laboratory has reported a similar recombination event involving A11-A12 and the a-locus allotypes. Our current breeding experiments are designed to test for recombination between a markers and both the A11-12 and A14-15 systems.

IgA and IgG were prepared from the colostrum of the recombinant rabbit (in collaboration with Dr. H. Reynolds, LCI). Both of the colostral immunoglobulins lacked the a1 and carried a2 and a3 as previously found for serum IgG. This was important because, certain models of the immunoglobulin genetic region would predict that IgA could have retained expression of the a1 type (present before recombination occurred).

Progeny of the recombinant rabbit and its sire have been bred and colonies of homozygous A1,14 (original chromosomal linkage group) and A2, 14 (recombinant chromosomal linkage group) rabbits have been developed. IgG from the sera of rabbits in these two colonies was prepared, as well as Fab, Fc, light and heavy chains of each. Each IgG was ^{125}I-labeled, reduced, alkylated, and run on calibrated Sephadex columns in guanidine in order to compare the molecular weights of the normal and recombinant heavy chains. In a series of experiments where labeled and unlabeled IgG preps were mixed, as well as by calculation of K_d values, no difference in molecular weights of the normal and recombinants chains was observed (H. Reynolds, M. Carta and R. Mage).

Fc-fragments of each IgG were digested with trypsin and the peptides separated. There were no unusual tryptic products from the Fc of the recombinant. The peptide with the amino acid interchange (T19, see above) was isolated from the Fc of the recombinant IgG and shown to have the additional threonine characteristic of A14 (collaborative studies with E. Appella, M. Carta).

Fd-fragments of each IgG were prepared which retained intact a-locus allotypic determinants of the parent molecule. Tryptic digestion of a1 Fd after blocking of lysines has yielded a series of peptides, one of which still retains a-allotypic determinants (studies in collaboration with E. Appella and M. Carta). The tryptic peptides are currently under study.

Rabbits are being bred and selected for allotype plus the ability to respond to pneumococcal vaccine with antibody of restricted heterogeneity. Anti-idiotype antisera were prepared which reacted with anti-SVIII antibodies of restricted heterogeneity in the sera of two rabbits, each from a different pedigree. No sharing of the idiotypes was detectable in antisera from close relatives of each donor. Antibody to SVIII with idiotypic determinants present during the first course of immunization of an individual rabbit reappeared during the second immunization series 12 months later. (Winfield, Pincus and Mage, 1972).

A rabbit partially suppressed for the expression of b5-K-type light chains when first immunized, produced anti-p-azobenzenearsonate antibodies at 8, 13, 34 and 41 months of age after each of four courses of immunization with an edestin-azobenzenearsonate conjugate. The antisera obtained after the second, third and fourth courses of immunization contained antibodies of limited structural heterogeneity. Light chains of b5 allotype were obtained from the purified antibodies. Amino acid sequence analysis of the first 21 residues at the amino terminus of the light chains indicated a single sequence for those from the third course antibody and a major sequence with limited heterogeneity from the second and fourth course antibodies. Anti-idiotype antisera were obtained by immunizing allotype-matched rabbits with anti-ars (D antigens) produced after each course of immunization. These sera were used to study the relationship between the anti-ars produced over a span of 33 months. Antibodies of limited heterogeneity produced 21 months apart had very similar idiotypes. Components with related idiotypes were also demonstrated to be present in the more heterogeneous earlier and later antibodies. We have attempted to subfractionate D antigens by preparative isoelectric focusing in the hope that some similar but non-identical components of antibody mixtures would be separated and identified. Our anti-idiotype antisera (anti-D) did not distinguish between four major fractions of anti-ars which separate on isoelectric focusing. (Experiments done in collaboration with J. Winfield, J. Pincus, E. Appella, C. Alexander, M. Freedman and others).

Sixty-four rabbits from our breeding groups with different genetic backgrounds were studied with respect to the concentration and affinity of anti-DNP antibody produced over the course of a year following a single immunization with DNP-BGG (collaborative study with G. Siskind, T. Werblin). The responses of males and females were identical. Rabbits in the colony with b6 allotype produced lower concentrations of antibody and b5 allotype was correlated with increased concentrations of antibody. Rabbits with a1 allotype produced high affinity antibody compared to the remainder of the population. During the immune response, individuals tended to maintain their relative position in the population with respect to affinity and concentration of antibody produced. The mean affinity of the population shifted towards high affinity during the period of observation and the standard deviation of the population with regard to affinity of the antibody produced, decreased with time. Taken together with previous observations, the data support the view that new types of antibody do not appear during the course of the immune

response. Limited data from the first breeding studies show that parents that produce high affinity anti-DNP antibody have offspring which also tend to produce high affinity anti-DNP antibody. Similarly, parents that produce relatively low affinity anti-DNP antibody also have offspring which tend to make low affinity anti-DNP antibody.

The proportion of peripheral blood lymphocytes with cell surface b5 immunoglobulin was compared to serum b5 IgG levels in allotype-suppressed and recovering b5 homozygous rabbits in order to determine the relation between lymphocyte membrane immunoglobulin and that of antibody secreted by differentiated antibody-secreting cells. For cell labeling, specific anti-b4 and anti-b5 reagents were prepared from antisera raised in b6 and b9 rabbits by cross-absorption on b4 and b5 IgG-coupled Sepharose columns. In suppressed rabbits with no detectable serum b5 no cells labeled with ^{125}I labeled or fluorescein conjugated anti-b5 allotype antibodies. Non-suppressed controls had up to 60% of cells labeled. During spontaneous recovery from suppression, lymphocyte membrane b5 appears before serum b5 is detectable and thus may represent a step in the differentiation of b5 secreting cells. In rabbits escaping from suppression, the proportion of peripheral blood lymphocytes with membrane-associated b5 returns to near normal range while the serum b5 levels remain abnormally low. It is possible that even in escaping animals, normal differentiation to b5 secreting cells is still partially blocked. The mechanisms underlying suppression continue to be under investigation. (Experiments in collaboration with M. Harrison and J. Davie).

In order to carry out all the studies outlined above, we breed and maintain large numbers of rabbits in our own colonies. We produce and characterize large volumes of anti-allotype antisera made with a variety of donor and recipient combinations, and purify normal and antibody immunoglobulins from large volumes of rabbit sera.

Significance to Biomedical Research and the Program of the Institute:

These studies help to define the genetic mechanisms which lead to the synthesis of highly specific antibody molecules and the nature of the genes which influence and control immune responses. Allotypic markers are providing valuable reagents for the study of cell surface receptors and the role they play in the differentiation of antibody producing cells. These investigations help delineate the chemical basis for specificity of protein antigens and antibodies, the nature of structural genes for immunoglobulin polypeptide chains and the complexities of mammalian genetic systems. The breeding studies which can be done with rabbits provide information applicable to the homologous human immunoglobulin system. In addition, rabbit allotypes are analogous in many respects to cellular isoantigens involved in transplantation, tumor antigens and other cell surface markers. An understanding of the factors which control genetic expression of these isoantigenic types is thus of broad fundamental interest.

Proposed Course of the Project:

We are continuing most of the studies described. The success of the cell labeling studies encourages us to pursue studies of the normal ontogeny and appearance of allotypes on cell membranes and in serum of rabbits. We hope to use reagents specific for a and b allotypes in future studies. Experiments are in progress which we hope will delineate the mechanism of allotype suppression. These include a number of approaches to the question of whether an actively sensitized cell is present in suppressed animals which mediates chronic suppression, and investigation of the effect of neonatal thymectomy on establishment of suppression. Similarly, initial successes in the isolation of peptides with allotypic determinants intact encourages us to pursue this important problem. We hope to localize the position in the heavy chain sequence of allotypic-determinant bearing peptides of the a-locus group. In our structural studies, we plan to use normal immunoglobulins and antibodies of restricted heterogeneity.

Honors and Awards:

Member of the Editorial Boards of Immunochemistry (1972-1975) and Federation Proceedings (1971-1972). Invited to join Allergy and Immunology Study Section (1972-1976).

Publications:

Mage, R.G.: Normal and altered quantitative expression of allotypes on light and heavy chains of rabbit immunoglobulins. Annals N.Y. Acad. Sci. 190: 203-211, 1971.

Mage, R.G.: Structural localization allelic exclusion and linkage relationships of rabbit allotypes. In Amos, Ed. Progress in Immunology, pp. 47-59, New York, Academic Press, 1971.

Appella, E., Chersi, A., Mage, R.G. and Dubiski, S.: The structural basis of the A14 and A15 allotypic specificities in rabbit immunoglobulin-G. Proc. Nat. Acad. Sci. 68: 1341-1345, 1971.

Landucci Tosi, S., Mage, R.G., Gilman-Sachs, A., Dray, S. and Knight, K.L.: Presence of the Fc-fragment allotypic determinant A15 on IgG from an allotype-suppressed Aa2 homozygous rabbit lacking the Aa2 determinants of the Fd fragment. J. Immunol. 108: 264-267, 1972.

Winfield, J.B., Martin, W.J. and Mage, R.G.: Immunization of neonatal rabbits with bovine serum albumin associated with allogeneic thymus cells-immune response and allotypic markers of antibody. Int. Arch. Allergy 41: 895-909, 1971.

Landucci Tosi, S., Mage, R.G. and Lawton, A.R.: Similar expression of a1 and a2 allotypes on IgG and IgM from heterozygous rabbits.

Immunochem. 9: 317-324, 1972.

Goldman, M.B. and Mage, R.G.: Purification and effectiveness of anti-allotype antibodies in suppression of rabbit immunoglobulin allotypes. Immunochem. In Press, 1972.

Winfield, J.B., Pincus, J.H. and Mage, R.G.: Persistence and characterization of idiotypes in pedigreed rabbits producing antibodies of restricted heterogeneity to pneumococcal polysaccharides. J. Immunol. In Press, 1972.

Serial No. NIAID - 147
1. Laboratory of Immunology
2. Bethesda, Maryland

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immunogenetics of Mouse Immunoglobulins

Previous Serial No: Same

Principal Investigator: Miss Rose Lieberman

Other Investigators: Project Subtitle II. Dr. B. Benacerraf
Harvard Medical School
Dr. Richard Asofsky
LMI, NIAID

Cooperating Units: None

Man Years:

	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	TOTAL
Total	2			2
Professional	1			1
Other	1			1

Project Subtitle I: Study of Immunoglobulin Determinants in Mice with Homologous Antisera to Determine the Genetic Control of the Structure of Immunoglobulin Molecules

Project Description:

Objectives:

1. To prepare homologous anti-allotypic and idiotypic (myeloma-specific) antisera highly specific for determinants on various parts of the different chains of mouse immunoglobulin molecules.

2. To study genetic control of immunoglobulin determinants.

3. To use genetically controlled immunoglobulin markers in studies of antibody synthesis.

4. To determine if identical molecules, based on biologic activity and identification of Fab determinants, can be found in myeloma proteins

from independently induced plasma cell tumors.

Methods Employed:

1. Preparation of immunoglobulin immunogens (Fc, Fab and light chains) from normal and plasma cell tumor bearing inbred mice.

2. Immunization of inbred strains of mice with specific normal or myeloma immunoglobulin molecules or fragments of immunoglobulin molecules (chains).

3. Identification of immunoglobulin determinants with homologous antisera using immunoelectrophoresis, Oudin and Ouchterlony gel diffusion methods, radioisotope tagging, precipitin inhibition method, hemagglutination inhibition techniques and immunoabsorbants.

4. Selective breeding of mice to determine linkage, allelism, independent assortment and crossover of genes. Production of coisogenic stains to propagate and isolate genetic markers in a known genetic environment. Chromosome mapping of genetic markers of immunoglobulin determinants with other genetic markers as coat color, etc., to facilitate ease of identification of immunoglobulin genetic markers and also to determine the location on the chromosomes of genes controlling specific immunoglobulin determinants.

5. Development of techniques employing immunoglobulin determinants as genetic markers in problems of transplantation, histocompatibility, transfusion reactions, immunization and antibody synthesis.

Major Findings:

Immunoglobulin determinants have been identified on the heavy chains of immunoglobulin molecules in the mouse. Six heavy chain γG (γ2a), three heavy chain γH (γ2b) and four heavy chain γA determinants have been identified. These determinants have been found to be closely linked in their genetic control. In addition, three other determinants which have thus far not been assigned to specific immunoglobulin molecules have also been identified. The 16 determinants are present in the Fc region (constant region) of the heavy chain. In addition two γF alleles have been identified by the differences in the electrophoretic mobility of their Fc fragments. Nine heavy chain linkage groups have been identified; six in inbred strains and three additional ones in wild mice.

Immunoglobulin determinants in other regions of immunoglobulin molecules, namely the Fab region, have also been identified for different classes of immunoglobulins. These have been identified with homologous precipitating antisera prepared with specific myeloma proteins. These antisera identify myeloma specific determinants (MSD) or idiotypic determinants that are restricted to the Fab region of the myeloma protein used as the immunogen.

Many of the myeloma proteins, specifically IgA's have been found to have biologic activity, i.e., they have been shown to combine with Pneumococcus C polysaccharide, Salmonella polysaccharide, dextran, 2,4-dinitrophenyl group (DNP), 2,4,6-trinitrophenyl groups (TNP), etc.

Recently, 8 plasma cell tumors, induced independently in genetically similar inbred mice (BALB/c), produced IgA myeloma proteins all of which combined with phosphoryl choline, which is found in the C polysaccharides of Pneumococcus and Lactobacillus. Homologous antisera prepared to these 8 functionally similar myeloma proteins showed that five of these (M299, H8, T15, S63 and S107) have a common antigenic determinant which was found to be on the Fab portions of the respective myeloma proteins. This common antigenic determinant was not found on the light or heavy chains alone but was only present on the Fab or on preparations of recombined light and heavy chains from the respective myeloma proteins indicating that this was a conformational determinant derived from the interaction of the light and heavy chains. Sequence studies of the amino terminus of the light chains of T15 and H8 indicate that the first 21 residues of both proteins are identical.

Homologous antisera to the three remaining functionally similar proteins (M167, M511 and M603) identified myeloma specific determinants that so far are unique to each separate myeloma protein used for immunization. Amino acid sequence studies of the light chain of two of these (M167 and M603) showed that there were differences between these two proteins as T15 and H8, and between each and beginning with the 9th residue.

Significance to Biomedical Research and the Program of the Institute:

It is difficult to study the genetic control of the diversity of antibody using normal antibodies since these are mixtures of many types of molecules. Myeloma proteins are pure molecular forms of immunoglobulins and these can be obtained by experimentally inducing plasma cell tumors in some strains of genetically defined inbred mice. In addition many of these myeloma proteins have been found to have antibody activity. Immunoglobulin determinants on both the common and variable regions of the heavy chains as well as conformational determinants involved in the antibody site provide tools to study the genetic control of the variability of antibodies. The finding of 5 identical myeloma proteins appears to indicate repetition of genetic mechanisms that control diversity of antibody production.

Project Subtitle II: Study of the Genetic Regulation of Antibody Synthesis

Project Description:

Objectives:

A. 1. Determine if the immune response to homologous antigens (γG (γ2a) myeloma proteins) is genetically controlled in mice.

2. Determine the effect of H2 types and immunoglobulin heavy chain genes of mice in the immune response to BALB/c γG myeloma proteins.

3. Map the immune response (Ir) genes in the H2 locus wherever possible.

B. 1. Determine agglutinin responses to SRBC and identify immunoglobulin heavy chain phenotypes in F_1, F_2 and backcross progeny of mice that had been genetically selected for high and low antibody synthesis for more than 16 generations.

Methods Employed:

A. 1. Inbred and congenic strains of mice of various H2 types from 5 different linkage groups of immunoglobulin heavy chains were immunized separately with BALB/c γG (γ2a) myeloma proteins.

2. The immune response based on antibody produced to the constant region heavy chain allotypic and the myeloma specific (Fab) idiotypic determinants on the immunizing myeloma protein was determined by passive hemagglutination and precipitation tests in agar gel.

3. Appropriate H2 recombinant strains were then selected and immunized separately with BALB/c γG (γ2a) and γA myeloma proteins to map the IrIgG gene relevant to the known IrIgA gene in the H2 locus.

B. 1. The immunoglobulin phenotypes, agglutinin titers to SRBC and levels of immunoglobulins were determined for F_1, F_2 and backcross progeny of mice genetically selected and bred for 16 generations for high and low antibody synthesis.

Major Findings:

A. 1. Immune responsiveness to IgG allotypes determined in 360 mice of various H2 types and from 5 different linkage groups of immunoglobulin heavy chains showed that:

Immune responsiveness to IgG allotypes in the mouse was controlled by an autosomal dominant immune response gene IrIgG linked to the H2 locus.

2. Chromosome mapping of the IrIgG gene relevant to the known IrIgA gene in the H2 locus showed that the IrIgG and the IrIgA were separate genes. The IrIgG is associated with the K region specificities to left of the Ss gene between the IrIgA and the Ss locus. Therefore, there are at least two Ir genes, one controlling the immune response to IgG and the other to IgA allotypes.

B. 1. The immune response gene for SRBC was found to be linked to the immunoglobulin heavy chain allotype. Immunoglobulin levels showed no linkage

to the immunoglobulin allotype but rather a simple pattern of inheritance.

Significance to Biomedical Research and the Program of the Institute:

The genetic control of the immune response is very complex and involves many genes. So far in the mouse both immunoglobulin heavy chain genes and H2 histocompatibility genes have been linked to the immune response genes. These genes provide markers to study gene products at the cellular level.

Publications:

Lieberman, R. and William Humphrey, Jr.: Association of H2 types with genetic control of immune responsiveness to IgA allotypes in the mouse. Proc. Nat. Acad. Sci., U.S.A. 68: 2510, 1971.

ROCKY MOUNTAIN LABORATORY

HAMILTON, MONTANA

July 1, 1971 through June 30, 1972

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PHS-NIH

Summary Statement

Rocky Mountain Laboratory, NIAID

Hamilton, Montana

July 1, 1971 through June 30, 1972

The continuing effort to curtail Governmental operations fortunately did not seriously affect our research program. Improving the efficiency of our Research Services and Maintenance Section released several positions when critical needs arose in the research program. The transfer of Dr. Lavelle to Bethesda left our slow virus research team without a virologist, and an effort should be made in the near future to restore virologic competence to this program. Likewise, the temporary assignment of Dr. Lodmell to Bethesda reduced our research effort in this area, but he should return with new ideas that should bolster our investigations. The imminent addition of Dr. Bruce Chesebro in July 1972 (now NIAMD) will also enhance our total research effort on the immunology of slow viral diseases.

Two of our staff were singly honored for their research accomplishments. Dr. John Bell was awarded the DHEW Meritorious Service Medal and Dr. Conrad Yunker was nominated for the annual Acarology Award presented by the Institute of Acarology, Ohio State University. Other evidence of peer recognition is our extensive collaboration with outside investigators and the number of guest workers who used our facilities. In addition to the usual visitors last year, we hosted scientists of the Division of Research Grants Tropical Medicine and Parasitology Study Section and the AIBS Advisory Group to the Office of Naval Research for one of their regularly scheduled meetings. Their interest in our facilities and programs was a prime factor in selecting this site.

Staff of this laboratory are primarily concerned with the microbiology, immunology, and control of animal-borne and arthropodborne diseases and other selected diseases, chiefly slow viral infections and tuberculosis, and with more basic research on various bio-immunologic responses to antigens and microbial constituents. Selected highlights of research, in part reported in 52 publications, are presented in the following sections.

Rickettsioses

After several years of investigations we have concluded that ticks are not likely involved in the ecology of epidemic typhus. Rickettsia prowazeki could not be recovered from more than 2000 ticks of 11 species collected in Egypt and Ethiopia, and all attempts have failed to permanently infect candidate species of African ticks by feeding them on rickettsemic guinea pigs and microtus. Transient infection was established in some species, but the organism was rarely passed transtadially and never transovarially. (Burgdorfer)

In studies on the dynamics of transovarial transmission of R. rickettsi, as revealed by electron microscopy, organisms in unfed nymphs were restricted chiefly to the ovarian external sheath, whereas in newly molted nymphs, rickettsiae were regularly seen in the ooplasm of oocytes, where they undergo intensive growth and invasion of cell nuclei during subsequent tick feeding. Related inquiry into the interactions between tick hemocytes and R. rickettsi, R. montana, and R. canada revealed rather uniform reactions of blood cells toward these organisms. Of various cell types, spherular hemocytes and plasmatocytes most frequently acquired infection, either by direct invasion or by phagocytosis of rickettsiae. (Brinton and Burgdorfer)

The biology of R. canada in various ixodid ticks and fleas has been studied experimentally to gain some insight into its natural ecology. This organism previously was found to be so pathogenic for Dermacentor andersoni and D. variabilis that these species could not serve as natural vectors. However, Amblyomma americanum tolerated the organism well and passed it transtadially and transovarially. The rat flea, Xenopsylla cheopis, a candidate natural vector, could be transiently and mildly infected but would not transmit infection to susceptible animals. (Burgdorfer)

Further investigations were made on the behavior of rickettsiae in mouse L cells and chick embryo cell cultures. For titrating infectivity, L cells were as sensitive as chick embryo cells to spotted fever and typhus groups, but less sensitive to R. canada and Coxiella burneti. All these rickettsiae regularly produced plaques in chick embryo cells, but the typhus group rarely did so in mouse L cells. Plaque size, appearance, and number were affected by diluent, temperature of incubation, number of cells planted, and centrifugation of inoculum onto cultures. Rickettsiae were most stable in brain-heart infusion; skim milk was the next best medium tested. In general, glutamate increased stability of suspensions, potassium salts were no better than sodium salts, and sucrose solutions were more detrimental than comparable mineral salt solutions. (Ormsbee, Wike)

The immune response to small doses of C. burneti was studied in persons who received 30 µg Phase I antigen 3 to 4 years before a skin-test dose of .01 µg Phase I antigen. When bled 35 days later, recipients had an anamnestic response, chiefly in IgM, which, when tested in pure state, had both Phase I and II agglutinins. This IgM also suppressed growth of C. burneti in challenged mice. (Ormsbee)

Data from previous studies indicated that the toxin neutralizing factor (TNF) in normal human serum, which selectively inactivates R. typhi toxin, was associated with β-lipoprotein. Rigorous attempts to separate TNF from β-lipoprotein have failed, and it has been impossible to distinguish between active TNF β-lipoprotein and that from other persons who possess no TNF. Gel diffusion tests suggest that if TNF is a β-lipoprotein, it is antigenically different from that detectable with the antiserums used, its concentration is below that detectable by immunodiffusion, or it may form nonprecipitating antibody complexes. (E. J. Bell, Munoz)

Tick-Borne Viruses

Continued investigations of ticks of seabirds revealed that these arthropods are responsible for maintaining and disseminating a wide variety of viruses. Last year 23 isolates were recovered from 61 pools of Ornithodoros sp. nr. capensis taken at nesting sites of the common murre, Uria aalge, near Cape Lookout, Oregon. Results of preliminary identification indicate a mixture of viruses, some of which are in the Hughes group and closely related to Punta Salinas virus. The 36 strains from Ixodes uriae, which were taken from seabird nesting sites in Oregon in 1970, have been classified into 4 antigenic groups: "B", Kemerovo, Uukuniemi, and Sakhalin. The group "B" viruses were shown to be Tuleniy virus and the Sakhalin group to be that virus. However, the Kemerovo-like viruses were not identical to any known members of the group and are believed to be new to science. This Kemerovo-group virus was also recovered from Ixodes uriae taken on St. Lawrence Island in 1971. This is the first recognition of a Kemerovo-group virus from ixodid ticks in the United States and the first reported presence of Sakhalin virus or a Uukuniemi-like agent in this country. (Clifford, Keirans, Thomas, Yunker)

The behavior of 68 arboviruses, chiefly tick-borne, was studied in Vero, BHK-21 and PK-H13 cell lines to develop a mammalian cell-culture system suited for assay and identification of this group of viruses. Vero cells were found to be superior to the other 2 in supporting growth and showing cytopathogenic effect (CPE) and/or plaques. Vero cells, in fact, were more sensitive than baby mice for recovering Colorado tick fever virus from ticks and from rodent and human blood specimens. However, some viruses that failed to replicate in Vero cells did so in one or both of the other 2 lines, sometimes with CPE and plaques. About 77 percent of 41 tick-borne viruses studied were detectable by their effect on one or more of the 3 cell lines used. (Yunker)

Vector-Pathogen Relationships

Significant progress has been made in the application of arthropod cell cultures for studying the biology of arthropodborne viruses and rickettsiae. A plaquing technique was developed that permits assay of certain arboviruses in Aedes albopictus cell monolayers. Of 68 viruses tested, 16 produced clearly defined plaques from 1 to 7 mm in diameter. All plaquing viruses were insect-borne and 10 were members of Casals' group B (flaviviruses). Six nonarboviruses and 4 rickettsiae failed to produce plaques. In related studies the growth of 1 nonarthropodborne virus, 8 tick-borne, and 5 mosquito-borne viruses was compared in 3 insect lines (Ae. albopictus, Ae. aegypti, Culex quinquefasciatus) and in explant cultures of Dermacentor andersoni viscera and hemocytes. In general, insect cell lines supported growth of insect-borne flaviviruses and tick cells supported growth of tick-borne flaviviruses. The specificity of orbiviruses, especially the Kemerovo group, was less marked and some crossed vector-class lines. (Yunker)

Further attempts were made to develop tick tissue cell cultures. The hemocyte cultivation technique was successfully adapted for use with the soft tick, Argas persicus. Attempts were made to galvanically stimulate cell division in primary tick explants, and in Antheraea and Ae. albopictus cell lines, but growth was retarded, or not affected. Treatment of cultures with ecdysone, a molting hormone, slightly stimulated Antheraea cells, but not others. Analytical studies of media were made to detect changes in constituents induced by cellular growth. Sugars were consumed in direct proportion to cell growth. So far, all attempts to develop continuous cell line cultures from ticks have failed. (Yunker, Parker)

Systematics of Arthropods

Renovations have been completed and the tick collection is now housed in an area from which all other laboratory activity has been excluded. The collaborative projects on ticks of Venezuela and the systematic revision of Ixodes subgenera have recently been completed. A new collaborative project with NAMRU-3 and the University of Wisconsin on ticks of Galapagos Islands is in progress. During the year, 10 new species of ticks were recognized and their descriptions published. (Clifford, Keirans)

Most of some 1500 collections of dermanyssoid mites of Venezuela and 225 collections of macronyssid mites of Surinam have been identified and a number of descriptive manuscripts have been published or are in preparation. Among this collection was a species of parasitic mite, taken from phyllostomatid bats, which contained developing filarial larvae of the subfamily Dipetaloneminae. The proven ability of parasitic mites and ticks to transmit certain species of dipetalonemine worms and the larval development seen in this mite suggest that mites are vectors of bat filariae. Among 20,000 specimens of chiggers in the Venezuelan collection, 50 undescribed species were recognized, of which half were in the pest chigger genus Eutrombicula. During the year, 8 new species and 1 new genus were characterized and their descriptions published. (Yunker, Brennan)

Abortive Rabies

Recovery after patent rabies has now been demonstrated experimentally in mice, dogs and cats. The development of a significant titer of neutralizing antibody in the brain or cerebro-spinal fluid (CSF) has been firmly established as a diagnostic criterion. At 90 weeks after recovery from infection, both dogs and cats retained significant titers in the CSF, but their serum antibody levels had markedly declined. (J. F. Bell)

Elucidating the basic mechanism involved in abortive rabies and finding means of manipulating the mechanism have received primary emphasis. By increasing the ambient temperature from 22°C to 35°C during the incubation period, the mortality of inoculated mice was markedly reduced, and it was proportional to the duration of exposure to the higher temperature. However, the rate of survival with sequelae was not affected. The simultaneous administration of 50 gamma of histamine sensitizing factor (HSF) from

Bordetella pertussis with rabies virus resulted in a tremendous increase, up to 5-fold, in survival of patently affected mice. A single dose of 50 gamma was near optimum and simultaneous administration was more effective than giving HSF before or after inoculation with virus. Preliminary results suggest that HSF increases the rate of development of antibodies to rabies virus, presumably aborting infection. Comparable tests with endotoxin, mycobacterial cell walls, cyclic AMP, and theophylline have not thus far yielded useful data. It would appear that the use of HSF to enhance the immune mechanism may have direct application in rabies prophylaxis in man and animals before or after exposure to rabies. (J. F. Bell, Munoz)

Psittacosis-Lymphogranuloma-Trachoma Group

Based on typing of isolates in several laboratories by the micro-immunofluorescence (micro-IF) test, the number of recognized serotypes of Chlamydia trachomatis are now 9 TRIC (types A-I) and 3 LGV (types I-III). In view of the reliability of the technique for typing isolates, the test was applied to the typing of antibody in human serums obtained from patients with arthritis and some thought to have venereal disease. The micro-IF test was not as sensitive as the radioisotope precipitation (RIP) test, but was 8 times more sensitive than the standard CF test. Over 80 percent of the V.D. Clinic serums were reactive in the RIP and micro-IF, but only 33 percent had CF antibodies. One-third of these serums showed specificity against one or more antigens, and in 5 of 8 persons, their serologic specificity corresponded with the serotype isolated from them. The immunoglobulin in the V.D. Clinic serums was determined with monospecific IgG, IgM, and IgA conjugated antiserums. IgG patterns of reactivity paralleled those of undifferentiated antibody. About 20 percent of the serums had IgM activity which was often type-specific (26 of 39 serums). This specificity was also found in the IgG and IgA, and when it occurred in several antibody classes, it was always to the same serotype. (Philip)

Previous research on the toxic effect of various chlamydia on mouse L cells had to be repeated because a mycoplasmal contaminant was eventually demonstrated in the cell line used for this work. Similar results were obtained in the mycoplasma-free system. However, a marked acceleration with the toxic effect was not recognized previously. When monolayers were inoculated with just enough chlamydia to infect all cells, inclusions were barely perceptible microscopically at 16 hrs and were mature at 36 hrs. When toxic concentrations were used as inocula, inclusions were 4 to 5 times larger than normal at 16 hrs and the host cells rapidly lysed before the inclusions matured. This phenomenon occurred with all strains of Chlamydia trachomatis. (Gerloff)

Centrifugal Chromatography in Silica Gels (Centri-Chrom)

Further modifications and adaptations of this technique were made to provide both a preparative and quantitative method for separating and assaying complex compounds soluble in nonaqueous solvents. The method for identifying the active compounds in marihuana and hashish reported

last year was praised by many as an improved qualitative analytical tool, but it lacked quantitative features. A system was devised to accelerate migration by pressure (80 to 100 psi) instead of by centrifugal force. By monitoring the effluent through a Chromatronix microflow cell at 280 nm in an LKB Uvicord coupled to a Heath recorder, it was possible to detect components as they were eluted. Fractions from each UV peak were then stained with Echtblausalz B and subjected to colorimetric analysis. The presence of 10 ug of Δ^9-tetrahydrocannabinol (Δ^9THC) could be detected with an accuracy of ± 5 percent by the colorimetric method. With this method it was possible to detect cannabinoid components, and their metabolites and/or conjugates, in the urine of a human subject who had ingested 40 mg hashish in divided doses 2 hours apart. By increasing the diameter of the column from 3 mm to 18 mm, working quantities of pure Δ^9THC were readily obtained. Previously, all the synthetic standards we had obtained were grossly contaminated with 5 to 9 other components. With working quantities of pure Δ^9THC now producible, scientists should be able to perform valid experiments of the effects of psychomimetically active components in hashish and marihuana. (Ribi, Smith, Parker)

Immune Prophylaxis of Mycobacterial Infections

Trials in monkeys on the duration of immunity induced by Mycobacterium bovis, Bovinus I, cell walls have almost been completed. The group of monkeys challenged at 12 months after intravenous vaccination have been autopsied, but an accurate evaluation of resistance must await detailed study of formalin fixed tissues. A preliminary scoring of gross lesions, however, would indicate a decrease in immunity at 12 months, with a significantly greater drop in cell-wall vaccinates than in living BCG vaccinates. Hypersensitivity to 250 tuberculin units given intrapalpebrally could not be correlated with protection. At 2 months postvaccination, when protection was excellent, only 7 of 34 cell-wall vaccinates reacted whereas at 12 months, when immunity had waned, 5 of 12 monkeys reacted. The pulmonary granulomatous response, which was prominent at 2 and 4 1/2 months, was still regularly detectable at 6 months, and only 3 of 8 monkeys autopsied at 8 1/2 months had normal lungs. In view of the short-term nature of immunity induced by the cell-wall vaccine and the adverse granulomatous response, further consideration of this product for a potential vaccine in man appears unwarranted. (Anacker, Ribi, RML; Brehmer, Robert-Koch Institute, Germany; Wolochow, Naval Biomed. Res. Lab., Oakland, Calif.)

Various approaches were utilized in further study of tuberculoimmunity in small laboratory animals. In search for a substitute for oil in the cell-wall vaccine, we tried to protect mice against aerosol challenge with alum-aggregated cell walls, but immunity could not be engendered, regardless of the route or number of doses. The development of pressure elution microparticulate gel chromatography enabled us to purify components of mycobacteria in sufficient quantities for conducting protection tests. With this procedure 3 components (P_1, P_2, P_3) of Dr. Kato's cord factor were isolated. Of those tested so far, only P_3, when recombined with NaOH-treated cell walls, conferred resistance comparable to that given by living

BCG. Dr. Kato found that this fraction markedly inhibited mitochondrial respiration and phosphorylation and had a higher toxicity for mice than any other cord sample he had tested.

Because previous studies had shown that a lipid extract of mycobacteria, when recombined with NaOH-inactivated cell walls, conferred tuberculo-immunity, the extract was thought to contain an adjuvant which enhanced response to the cell wall immunogen. To confirm this hypothesis we tested the adjuvant activity of various mycobacterial combinations on other antigens. Cell walls enhanced antibody formation in mice to egg albumin but cells and lipid extract did not. Cell walls but not the combination promoted delayed hypersensitivity to DNP-human serum albumin in guinea pigs. In contrast, the combination, but neither component alone, induced footpad sensitivity in mice. Finally, the combination was more effective than either component alone in inducing experimental allergic encephalitis. These results suggest that different compounds are responsible for activity in different assay systems and that no single "adjuvant" exists. (Anacker, Meyer, Smith)

Adjuvant Action of Mycobacterial Cell Walls in Tumor Immunity

Since some serious problems are associated with the use of living Mycobacterium tuberculosis, BCG, to stimulate tumor immunity, a collaborative study with NCI was undertaken to determine whether BCG cell walls would enhance immunity to a syngeneic transplantable hepato-carcinoma in strain 2 guinea pigs. Tumor growth was suppressed if 10^6 tumor cells were inoculated with 75 ug BCG cells attached to oil droplets but not if tumor cells were given with BCG cell walls alone, oil droplets, or oil droplets and BCG cell walls separated from each other. BCG cell walls (300 ug)-oil droplet complex injected into established tumors caused them to regress and prevented the development of metastases. Similar treatment with M. smegmatis cell walls was ineffective. Results of further studies showed that sodium hydroxide treated BCG cell walls, from which lipids had been removed, were highly effective, but the lipids mixed with oil droplets were ineffective. It was also shown that the immunity induced by BCG cell walls was indeed systemic and not related to the site of adjuvant injection. Unquestionably, these results will have a tremendous impact on immunotherapy of cancer in man. (Ribi, RML; Zbar, Rapp, NCI)

Molecular Biology of Endotoxins

In further investigations on endotoxin tolerance, some parameters other than pyrogenicity in rabbits and the activities of synthetic double-stranded RNA (poly I-poly C) in this system were studied. In rabbits, no significant resistance to lethal challenge developed within 24 hrs after preparation with a single dose of homologous or heterologous endotoxin, and only those vaccinated homologously were resistant after 8 days. Generally mice become resistant to endotoxin after a series of preparative inoculations, but RML mice did not develop much resistance after a single prior exposure, regardless of dose or interval between preparation and challenge. They

were difficult to protect passively with hyperimmune serums, and it appeared that protection depended on antibody to determinants other than O antigen. Cross tolerance between endotoxin and poly I-poly C did not occur. However, a single preparative dose given 24 hrs before a standard homologous challenge with poly I-poly C produced tolerance to the pyretic effect, but the preparative dose had to be 20 to 50 times more, in terms of activity, than would be required in an endotoxin system. The findings of the past year indicate that tolerance must be evaluated in terms of specific effects in specific species and that the broad mechanisms of induced resistance so far identified do not explain the complexity of observed results. (Milner and Ribi)

The essential features of composition and structure of endotoxin have heretofore been studied chiefly by hydrodynamic techniques. In a collaborative project this year with the U.S. Army, we studied an aqueous-ether extract of Salmonella enteritidis of high biologic activity by viscometry, osmometry, and light-scattering techniques. The results showed the endotoxin to exist in polydisperse, elongated particles of high molecular weight, which were more aggregated when dissolved in water than in dimethylformamide. These results are compatible with those we had obtained by measurements of sedimentation and diffusion. Examination of lyophilized preparations under the scanning electron microscope revealed that endotoxin had further aggregated into fibrous structures with marked resemblances to cellulose and certain synthetic fibers. (Milner, RML; Cross, U.S. Army Materials Research Agency, Watertown, Mass.)

Bordetella Pertussis Antigens

The low concentration of γE-like antibody in the serum of mice immunized with a mixture of protein antigens and Bordetella pertussis extracts has complicated separation, purification, and characterization of this immunoglobulin. Although specific antiserum to γE-like antibody has been produced in guinea pigs, as judged by the neutralization of the 72-hr PCA, these serums were nonreactive in precipitation or radioimmunodiffusion tests. The selective response in the γE class presumably is due to the adjuvant action of the histamine sensitizing factor (HSF). Since HSF is also responsible for the accelerated immune response in abortive rabies, it becomes even more important to obtain this fraction in pure form. In previous attempts to purify HSF, solubility decreased as contaminants were removed. When HSF, extracted from acetone-treated cells with 0.05 m sodium pyrophosphate in 1 M NaCl at pH 8.5, was passed through hydroxylapatite columns, a soluble product was obtained that could be further purified by starch block electrophoresis. Recovery was poor and further attempts are being made to improve the yield. (Munoz)

Previous findings have strongly suggested that the biologic response to HSF is mediated, in part, by the adrenal glands. Hence, much effort was directed toward developing a system for quantitation of catecholamines and steroids in mouse adrenals. An assay procedure utilizing 2-step chromatography, internal standards of ^{14}C-epinephrine and ^{3}H-norepinephrine, and

quantitation by fluorescence gave good results with known standards in the range of 0.1 to 10 ug/ml. However, when the procedure was applied to catecholamine extracts from mouse adrenals, higher values than those published were obtained. The instability of radiolabeled catecholamines appeared to be responsible for the discrepancy and the problem has not been fully resolved. (Bergman)

Slow Viral Diseases

Additional data obtained the past year now provide a clearer picture of the pathogenesis of naturally occurring scrapie in sheep. Early growth of virus (10^{-2} to 10^{-3}) definitely occurs in visceral and peripheral lymph nodes and other organs containing lymphoid tissue (tonsil, spleen, ileum and upper colon, nasal mucosa). These results suggest that virus enters the host via the nasopharynx or lower gastrointestinal tract, spreads to the regional lymph nodes, replicates, and then spreads to other lymph nodes and spleen. After many months virus appears in the central nervous system, where it slowly increases until signs of disease appear. Though the familial occurrence of scrapie suggests vertical transmission of virus (in utero), we have not been able to isolate virus from fetuses or newborn lambs. However, we did recover virus from clinically normal yearling and 2-year-old sheep which were progeny of ewes in high-risk Suffolk family lines. It would appear that the virus is acquired during the first 4 months of life, when there is close contact between ewe and lamb. (Hadlow, Race, RML; Klingsporn, USDA)

Other workers have reported that sapphire and pastel mink are both highly susceptible to the Utah strain of Aleutian disease (AD) whereas, working with the Pullman strain, we have found that only 30-50 percent of pastels will develop AD, even after inoculation with large amounts of virus. When RML pastel mink were inoculated with the Utah strain, all that received 220 mu or 100 mu Millipore filtrate developed the disease, but none of those receiving the 50 mu filtrate became ill. Furthermore, both pastels and sapphires receiving the coarser filtrates developed signs of a transient acute disease 2-3 weeks after inoculation, which was never seen with the Pullman isolate. These observations suggest that the Utah strain may be contaminated with another pathogen which was removed by the 100 mu filter. These preliminary results suggest that the Utah strain is more virulent for pastels. Comparative titrations now in progress should provide a better understanding of the differences observed, particularly the failure of the Utah isolate to pass the 50 mu filter. (Hadlow, Race)

Allergy and Immunology

Various parameters of the immune response of mice were investigated. Confirming the work of others, we also found that mice developed delayed hypersensitivity to methylated bovine serum albumin (MBSA). Mice sensitized with 2 injections of MBSA given subcutaneously 7 days apart responded with delayed skin and footpad reactions in the absence of visible 4 hr reactions. However, the macrophage migration inhibition test performed with peritoneal macrophages of mice known to have delayed skin or footpad

reactions was invariably negative, regardless of whether they received MBSA, human serum albumin, or mycobacterial oil-cell-wall vaccine. (Munoz)

Since the thymus presumably is involved in the γE-like antibody response of normal mice to protein antigens mixed with B. pertussis extracts (SE), the ability of nude mice (thymusless) to respond similarly was studied. These mice, after inoculation with egg albumin, keyhole limpet hemocyanin, or SE, did not produce 2-hr PCA antibody ($7S_{\gamma 1}$), 72-hr PCA antibody (γE-like), nor hemagglutinating antibodies, but they did respond to Escherichia coli endotoxin; the thymus containing litter mates responded as well as or better than CFW mice with the production of $7S_{\gamma 1}$ and γE-like antibodies, and perhaps also with $7S_{\gamma 2}$, γM and γA. By Schultz-Dale test, we showed that the uteri of nude mice previously immunized to different antigens failed to contract in the presence of specific antigen, whereas normal litter mates and CFW mice did so. (Munoz, RML; Jutila, Reed, Montana State University)

During continued studies on the immunoglobulin classes of rodents, 3 electrophoretic variants (phenotypes) among $7S_{\gamma 1}$ globulins of peromyscus were defined and shown to be due to differences on the $7S_{\gamma 1}$ Fc piece (H chain). By breeding experiments, this polymorphism was shown to be controlled by at least 2 allelic $7S_{\gamma 1}$ H chain genes. Allotypy of immunoglobulins expressed by electrophoretic variation is rare—the only other known example is the $7S_{\gamma 1}$ globulin in Mus musculus, a distant relative of peromyscus. The genetic event responsible for this polymorphism probably occurred in a common ancestor, and therefore a comparable $7S_{\gamma 1}$ polymorphism should occur in other rodents. The immunoglobulins of Calomys callosus are now being studied for this and other properties. (Coe)

In studies on the metabolism of immunoglobulins in the hamster, it was interesting to note that mineral oil emulsions given intraperitoneally, without antigen, caused up to a 10-fold increase in serum IgM concentration. The increase was due to an accelerated synthetic rate and not a decrease in metabolism. The biological half-lives of IgM, $7S_{\gamma 2}$, and IgA were 24, 72, and 20 hours, respectively. (Converse and Coe)

Serial No. NIAID-170
1. Rocky Mountain Laboratory
2. Office of the Director
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Office of the Director

Previous Serial Number: Same

Principal Investigator: H. G. Stoenner, Director

Other Investigator: R. N. Philip, Assistant Director

Cooperating Units: None

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	43 2/12			43 2/12
Professional:	1 2/12			1 2/12
Other:	42			42

Project Description:

This project is primarily concerned with the general supervision of research programs, establishing program emphases, and procuring and allotting necessary resources to achieve program goals. Personnel engaged in general research services, such as clerical assistance, animal production, library and graphic arts services, general administration, and maintenance of buildings and grounds are included in this project, but others engaged in glassware and media preparation and care of animals under experimentation, which are direct support services, are allotted to each project in proportion to support given.

Honors and Awards:

Dr. H. G. Stoenner

Continued as a Reviewer for American Journal of Veterinary Research.

Continued service on the Committee on Environmental Health of the Montana State Advisory Council on Comprehensive Health Planning.

Continued service on the Board of Directors of the Montana Chapter of the American Cancer Society.

Serial No. NIAID-171
1. Rocky Mountain Laboratory
2. Rickettsial Diseases and Medical Entomology Sections
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Rickettsiae and rickettsial infections

Previous Serial Number: Same

Principal Investigators: E. J. Bell, W. Burgdorfer, and R. A. Ormsbee

Other Investigators: L. P. Brinton, R. K. Gerloff, L. E. Hughes, J. J. Munoz, M. G. Peacock, L. A. Thomas, and D. A. Wike

Cooperating Units: Dr. J. C. Cooney, Tennessee Valley Authority, Muscle Shoals, Alabama
Dr. P. Fiset and Dr. C. L. Wisseman, Jr., University of Maryland School of Medicine, Baltimore
Dr. J. L. Lancaster, Jr., Department of Entomology, University of Arkansas, Fayettesville
Dr. Bennett Elisberg, Department of Rickettsial Diseases, WRAIR, Washington, D.C.
Dr. Emilio Weiss, Department of Microbiology, National Naval Medical Center, Bethesda, Maryland
Dr. Ronald Silberman, Louisiana State University Medical Center, Shreveport, Louisiana
U.S. Army Medical Research Institute, Fort Detrick, Md.

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	11 11/12			11 11/12
Professional:	4 7/12			4 7/12
Other:	7 4/12			7 4/12

Project Description:

Objectives:

1) Development and improvement of serologic and microbiologic methods useful in research on rickettsial diseases, 2) development of improved diagnostic reagents, and tests, and rickettsial vaccines (both killed and attenuated), 3) studies of ecology and epidemiology of rickettsioses in selected areas of the world including the United States, 4) antigenic analyses of natural groups of pathogenic rickettsiae, i.e., spotted fever group, typhus group, 5) development of tissue culture techniques and their application in study of rickettsiae, 6) attempts to isolate and identify a nonantibody factor in normal human serum which neutralizes the toxic effect of Rickettsia typhi, 7) study of parameters of experimental rickettsial infections in animals, embryonated eggs, and tissue cultures, 8) study of immune response in animals and man to rickettsial antigens.

Methods employed:

Standard methods in rickettsiology have been employed, utilizing guinea pigs, mice, embryonated eggs, agglutination and complement fixing tests. Tissue cultures of various cells have been employed. Standard chromatographic, electrophoretic and immunologic techniques, plus improved modifications of these as well as improved chemical techniques, have been used for isolation and characterization of serum components.

Major findings:

1. A standard method for producing purified, particulate rickettsial antigens has been developed. This method does not involve the use of ether, takes less time than former methods, and works with all species of rickettsiae tested; the final products can be used interchangeably in both microagglutination (MA) and complement fixation (CF) tests.

2. An ecological study of "Land Between the Lakes", a recreational area in the TVA region, revealed the presence of antibodies against R. rickettsi in 32 of 58 cottontail rabbits, 5 of 36 woodchucks, 3 of 155 raccoons, 1 of 3 fox squirrels, 4 of 39 voles. Typhus-group CF antibodies were found in 4 of 58 cottontail rabbits, 1 of 155 raccoons, 1 of 59 gray foxes, 1 of 36 woodchucks, 1 of 3 fox squirrels, and 1 dog. Examination of ticks for R. rickettsi showed infection only in Dermacentor variabilis, of which 6.2% of 886 were infected. Spotted fever group rickettsiae were not found in D. albipictus, Amblyomma americanum, Haemaphysalis leporis-palustris or in smaller numbers of various Ixodes spp.

Preliminary findings in a similar ecologic study in Arkansas indicate the presence of R. rickettsi in A. americanum and I. scapularis as well as in D. variabilis.

3. A nonantibody toxin neutralization factor (TNF) in normal human serum which selectively neutralizes the toxic action of R. typhi has been found to be closely associated with highly purified β-lipoprotein and indeed, not separable from it in spite of rigorous attempts to do so. Degradation of the purified β-lipoprotein also destroys TNF. However, it has not been possible by chemical, physical, or serological means to distinguish between β-lipoprotein that possesses TNF and that from other individuals which does not. Nor can the presence of TNF be related to the concentration of β-lipoprotein in the plasma. Gel diffusion tests suggest that if TNF is a β-lipoprotein, it is antigenically different from the β-lipoprotein detectable with the antisera employed. It may be present in purified β-lipoprotein preparations in concentrations too low for detection by immunodiffusion, or it may form nonprecipitating antigen-antibody complexes.

TNF associated with β-lipoprotein does not fix complement in the presence of typhus antigens, does not inhibit typhus-specific CF reactions, and does not agglutinate typhus rickettsiae. These and other tests demonstrated conclusively that TNF in normal human serum is not an immunoglobulin.

4. Very small booster doses of Coxiella burneti (Q fever) antigen given to individuals originally immunized with a single 30 μg dose of Phase I antigen, skin tested 3 to 4 years later with 0.01 μg Phase I antigen, and bled 35 days after skin test caused anamnestic response of IgM specific antibodies. Whole serum titrations showed high Phase I agglutinin values, very low Phase II agglutinin values and no CF antibody. By fractionating the serum, we found most of the activity in the IgM fraction which now possessed both Phase I and Phase II agglutinins. Mouse neutralization tests with various purified serum fractions indicated that the IgM which possessed specific agglutinins also suppressed the growth of C. burneti in challenged mice.

5. Parameters of rickettsial infections have been studied in mice, guinea pigs, embryonated eggs, primary chick tissue culture and related to the direct rickettsial count of the infectious material. R. prowazeki, R. typhi, R. rickettsi, R. sibirica, R. conori, R. canada, C. burneti, Phase I and C. burneti Phase II have been studied in this manner. Reproducible ratios among ID_{50} endpoints, plaque-forming units, and direct rickettsial counts have been established. Highest values of infectivity were given most consistently by plaque forming unit (PFU) values in cultures of primary chick embryo cells.

6. Rickettsial growth in tissue culture and factors affecting plaque formation have been studied in mouse L cells and primary chick embryo cell cultures. A variety of media, which have been employed during purification of live rickettsiae or during metabolic studies, have been examined for their effect on subsequent plaque formation.

For titration of infectivity, L cells are about as efficient as primary chick embryo cells with the spotted fever and typhus groups. L cells are less susceptible to infections with R. canada and C. burneti. Cytopathic effects in L cells were greatest with the spotted fever group, less with C. burneti and least (generally absent) with members of the typhus group.

Rickettsial plaque size, appearance, and number were influenced by the diluent employed, temperature of incubation, number of host cells initially planted, and centrifugation of inoculated cultures. Temperature and diluent were the most critical parameters. Brain-Heart Infusion (Difco) was the best of all diluents tested. Plaque formation was unaffected by genetic background of host (chick embryo) cells, volume of inoculum, rapid freezing and thawing of inoculum, temperature and length of incubation before nutrient overlay.

Examination of a wide variety of media commonly employed in metabolic studies, storage, or titrations of infectiousness has shown that rickettsiae, even at 0°C, rapidly lose their plaque-forming ability when suspended in them. The effect is more marked at 26°C. Only Brain-Heart Infusion did not cause both a drop in PFU and a delay in plaque formation. Skim milk was the next best medium found. In general, glutamate increased the stability of suspensions, potassium salts were not significantly better than sodium salts, and sucrose solutions were more detrimental than comparable mineral salt solutions.

7. WHO Reference Center function. The RML is participating in a world-wide program with 6 other reference laboratories which is designed to compare the serologic testing of coded serum specimens in the various reference laboratories. Specimens for this program originate in the participating laboratories. Any commonly accepted serologic procedure may be used. RML will titrate specimens by two different CF procedures and by microagglutination test.

As a WHO Center function, RML last supplied on request to domestic and foreign applicants 20 antigen preparations for agglutination or CF tests against R. rickettsi, R. akari, R. conori, R. canada, R. prowazeki, and R. typhi; 8 standard antisera against C. burneti, R. rickettsi, R. typhi, and R. prowazeki; 16 live seeds including C. burneti, R. rickettsi, R. typhi, and R. prowazeki. The requests came from 11 different laboratories.

The Serology Unit has continued to participate in the CDC Proficiency Testing Program as a Referee Testing Laboratory. It has also continued to perform standard serologic tests for rickettsial antibodies on specimens submitted by physicians, veterinarians, hospitals, clinics, State Health Departments, foreign domestic investigators, and to exercise surveillance of RML personnel for serologic evidence of rickettsial disease.

8. A collaborative project with Dr. E. Weiss of NMRI and Dr. B. Elisberg of WRAIR is aimed at establishing the guanine:cytosine ratio in the DNA's of R. canada and members of the spotted fever and typhus groups.

9. See Project NIAID-186 for related research on rickettsioses.

Significance to biomedical research and the program of the Institute:

The new standard method for producing purified particulate antigens works well with every pathogenic rickettsia so far tried. The antigens produced are stable, do not become anticomplementary on standing, and can be used interchangeably in agglutination and CF tests.

Studies of immune response in Q fever vaccinees following administration of skin test doses of antigen have shown that the anamnestic response may be IgM, instead of IgG as expected, that this IgM is protective in nature and that its presence may be completely missed if CF tests only are relied upon. In addition the antibody response has a prominent Phase I antibody character which is typically not seen following either clinical Q fever or primary immunization.

The identification of a TNF which very clearly is not an immunoglobulin is the first instance in rickettsial disease of an apparent protective mechanism of this nature. This finding is an unexpected addition to defense mechanisms against rickettsial disease.

Establishment of relationships between absolute numbers of rickettsiae, plaque forming units, and ID_{50} in animals and embryonated eggs permits quantitative comparisons of rickettsial groups, species and strains. It is now possible to detect and measure changes in virulence and to assess the degree of biologic variability existing within natural antigenic groups.

Development of the tissue culture and plaque technique has given us another parameter by which to compare species and strains of rickettsiae, a good method for establishing clonal lines of rickettsiae, another method for primary isolation of rickettsiae from natural sources and a technique whereby genetic variation can be studied. It has already proved useful in making assessments of suspending and holding media.

Studies on ecology of rickettsial disease in nature are essential to the long-term control of human disease. The studies in "Land Between the Lakes" and in Arkansas have revealed the presence of spotted fever in a variety of animals and ticks in recreational areas which are heavily used every year. Such studies not only give additional insight into the natural disease, they also can help the managers of such areas in efforts to prevent recreationally acquired human disease.

The guanine: cytosine ratios in DNA's of rickettsiae can be interpreted as one of the definitive criteria of relatedness among species. The current investigations, now virtually completed, will provide information on the degree of relatedness of R. canada to the spotted fever and typhus groups.

Proposed course of project:

Additional cooperative ecological studies of spotted fever are planned or now underway. These include a study with Dr. J. L. Lancaster, Jr., Department of Entomology, University of Arkansas, relating to prevalence of spotted fever infected ticks in selected areas in Arkansas, a study of the relationship of cottontail rabbits in Montana and Tennessee to the maintenance of spotted fever in nature, and a study with Dr. J. Cooney, Environmental Biology Branch, TVA, on the role of the tick Haemaphysalis leporispalustris as a vector of spotted fever in Alabama.

Attempts will be made to detect immunologic differences in lipoprotein fractions from TNF positive and negative sera. Studies will be initiated on the mechanisms in mice of toxic death caused by suspensions of live rickettsiae.

Further studies are planned on the immune response to rickettsial antigens with regard to the types of immunoglobulins involved, relationship of particular antibodies to immunity and the role of incomplete or blocking antibodies.

A cooperative study on recrudescent Q fever infection in pregnant women is underway with Dr. Paul Fiset, University of Maryland School of Medicine, and Dr. Ronald Silberman, Louisiana State Medical Center. We hope to identify C. burneti in the placentas from women who have previously suffered Q fever and to follow possible immunologic and clinical sequellae in their children.

Antigenic variation in rickettsiae will be studied in tissue culture systems, particularly with reference to phase shifts in C. burneti. Tissue culture techniques will also be employed in studies on improved suspending and preserving media for rickettsiae, as well as in a continuing study of changes in rickettsial plaques which may be related to biological changes in the rickettsiae per se. A collaborative project with the U.S. Army Medical Research Institute of Infectious Diseases constitutes an examination of the Russian M/44 strain of attenuated C. burneti, currently used as a living vaccine in the U.S.S.R. We will examine its biological characteristics, genetic stability and antigenic character.

Honors and Awards:

Dr. E. J. Bell

Awarded the DHEW Meritorious Service Medal, July 1971.

Dr. R. A. Ormsbee

Presented with gold medal as a founder of the Tissue Culture Association in 1946 at Hershey, Pennsylvania.

Publications:

Fiset, P., Wike, D. A., Pickens, E. G., and Ormsbee, R. A.: An antigenic comparison of strains of Coxiella burneti. Acta Virol. 15: 161-166, 1971.

Peacock, M. G., Ormsbee, R. A., and Johnson, K. M.: Rickettsioses of Central America. Amer. J. Trop. Med. 20: 941-949, 1971.

Ormsbee, R. A., Peacock, M. G., Bell, E. J., and Burgdorfer, W.: Experimental infections of lambs with Rickettsia prowazeki. Amer. J. Trop. Med. 20: 950-957, 1971.

In press:

Wike, D. A., Tallent, G., Peacock, M. G., and Ormsbee, R. A.: Studies of the rickettsial plaque assay technique. Infect. Immun.

Burgdorfer, W.: Rickettsioses (Introduction, Rocky Mountain spotted fever, Boutonneuse fever, North Asian tick typhus, Queensland tick typhus, rickettsialpox, murine (flea-borne) typhus fever, scrub typhus, Q fever). In Hubbert, W. T., McCulloch, W. F., and Schnurrenberger, P. R. (Eds.): Diseases Transmitted from Animals to Man (ed. 6).

Serial No. NIAID-173
1. Rocky Mountain Laboratory
2. Zoonoses Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Selected zoonoses of regional importance

Previous Serial Number: Same

Principal Investigator: J. F. Bell

Other Investigators: R. K. Gerloff, B. M. Hestekin, G. J. Moore, J. J. Munoz, Cora R. Owen, and H. G. Stoenner

Cooperating Units: Dr. Myron A. Andrews, Dept. of Veterinary Medicine, North Dakota State University, Fargo
Dra. A.M.O. de Diaz and Dr. E. Fuenzalida, Centro Panamericano de Zoonosis, Ramos Mejia, Prov. de Buenos Aires, Argentina
Dra. Maria Sancho, Instituto Pasteur de Avellaneda, Buenos Aires, Argentina
Dr. M. Gonzalez, Instituto Pasteur de Buenos Aires, Argentina
Dr. H. Delpietro, Servicio de Lucha Sanitaria, Argentina
Dr. Leslie Page, National Animal Disease Laboratory, Ames, Iowa

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	4 4/12			4 4/12
Professional:	2 7/12			2 7/12
Other:	1 9/12			1 9/12

Project Description:

Objectives:

Elucidation of the ecology of Francisella tularensis, rabies virus and other zoonotic microorganisms and investigation of the pathogenesis and immunology of some zoonoses.

Methods employed:

Field work in ecology and routine laboratory techniques of microbiology, entomology and immunology are employed as necessary for surveillance of regional outbreaks of disease.

Major findings:

1. Epizootiology of tularemia. F. tularensis has been isolated intermittently from one local stream for years. We are now attempting to pinpoint the source and to test the hypothesis that animals affected with infectious nephritis, with shedding of organisms in the urine, are responsible for contamination of water. Infectious water has been traced to a boggy tributary, and soil at the head of the tributary is contaminated with F. tularensis. Samples are being taken over a larger area and from surface and deeper soil. Animals obtained by trapping in that area will be tested for infectious urine.

Chronic tularemic nephritis with shedding of organisms has been produced in voles and mice in the laboratory and its pathogenesis is being defined.

Little additional work has been done on the interrelationships of several organisms of the Pasteurella-Yersinia-Francisella group because of the absence of Dr. Cora Owen (on sick leave).

2. Rabies.

a. Nonfatal rabies infection in mice. The mechanism of abortion of infection is emphasized. Two leads have developed recently. One concerns the effects of temperature on the outcome of infection. In repeated experiments, mortality has been reduced by more than 50 percent when the ambient temperature was increased from 22° to 35°C during the incubation period. The reduction was proportional to duration of exposure to higher temperatures, regardless of when (early or late) exposure occurred. This finding implies that rabies virus is vulnerable throughout the incubation period. However, there was no increase in the rate of survival with sequelae, which suggests that central nervous system (CNS) infection, once established, is not inhibited by the increased somatic temperature that accompanies high ambient temperatures.

The other lead stems from studies on the effects of the histamine sensitizing factor (HSF) of Hemophilus pertussis on the course of infection. Fifty gamma of HSF administered with the virus caused up to 5-fold increase in survival of patently affected animals, i.e., inhibition of disease after CNS infection was established. Fifty gamma of HSF was the near-optimum dose and its simultaneous administration with virus was more favorable than its administration before or after virus. It also appears that repeated doses

do not further enhance survival. The efficacy of HSF by various routes is being determined. Preliminary results suggest that HSF increases the rate of development of antibodies to rabies virus, presumably aborting infection. This finding is in accord with our evolving concept of CNS immunity. Comparable tests with endotoxin, mycobacterial cell walls, cyclic AMP and theophylline have, thus far, not yielded useful results.

b. Nonfatal rabies in dogs. A bitch that survived rabies and recovered from paralysis gave birth to 8 pups. Blood was taken from the pups before suckling and rabies antibody was present in all. This bitch, 3 other dogs, and a cat which recovered from rabies, have been kept for studies of the rate of decline of antibodies in serum and cerebrospinal fluid (CSF). Antibody levels have declined markedly 90 weeks after infection, but the CSF titer, which we consider indicative of abortive CNS infection, is adequate for retrospective diagnosis and should remain adequate for a long time. The other aspect of CNS immunity which can be studied in this system is the permeability of the blood/brain barrier to humoral antibodies during infectious and allergic encephalomyelitis. Studies to date indicate that antibodies to other disease agents do not enter the CNS as a result of rabies encephalitis in dogs. Correct interpretation of the significance of CSF antibody titers depends upon adequacy of information regarding permeability under diverse conditions. We feel that application of CSF immunology has been inadequately explored and utilized in the practice of medicine.

3. Mycoplasmal abortion. In the spring of 1969, a severe outbreak of the so-called "weak-calf disease" occurred in the Bitterroot Valley. The syndrome was considered by veterinarians to be epizootic bovine abortion, a chlamydial disease. Two agents (designated #70 and #71) lethal for chick embryos after yolk sac inoculation were isolated from calves, but neither was a chlamydia. Independently at the Veterinary Research Laboratory in Bozeman, an embryo-lethal agent also was recovered from the tissues of calf 3222 that yielded isolate #71. Both agents from this calf were sent to Dr. Leslie Page at the National Animal Disease Laboratory (NADL), Ames, Iowa, where the isolates were identified as identical mycoplasmas and designated as strain 3222.

Dr. M. L. Frey, a specialist on mycoplasma at NADL, has found strain 3222 to be antigenically unrelated to other common strains of mycoplasma from bovine, avian or human sources. Interestingly, an isolate recovered in 1964 by Dr. Robert Philip from tissues of a calf having the "weak-calf disease" also has been determined to be a mycoplasma and indistinguishable from strain 3222. In June 1969, blood samples were collected from 186 cows in a herd that suffered heavy calf-loss the preceding spring. Twenty-two percent of the sera were positive in complement fixation tests against antigen prepared from strain 3222. Titers ranged from 1:4 to 1:48 and 80 percent were 1:16 or higher.

The mycoplasma is believed not to be the direct cause of the "weak-calf syndrome", but studies are underway at Ames to determine its effect on pregnant cows.

4. Leptospirosis. One of the problems in the diagnosis and control of leptospirosis is the variable reliability of diagnostic antigens commercially available. The Leptospirosis Committee of the U.S. Animal Health Association proposed last year that only antigens licensed by the Department of Agriculture be used for the serodiagnosis of this disease. Standard plate antigens from pomona, icterohaemorrhagiae, canicola, hardjo, and grippotyphosa serotypes have been prepared for use by the Department of Agriculture for controlling the quality of antigens they license.

5. Service activities. Pasteurella multocida was isolated from swans dead or dying on a National Wildlife Refuge, from the mouth of a cat that bit a laboratory worker and from a dead robin. F. tularensis was isolated from a wild beaver, Clostridium chauvoei was found in Dermacentor andersoni from a dead elk, and Salmonella typhimurium was found in 2 more English sparrows (3 the previous year) coincident to a rather widespread decline in song bird populations. Specimens from a goat submitted for bacteriology by the "slow virus" section yielded one culture of Corynebacterium pseudotuberculosis.

Eighteen bats were examined and rabies virus was isolated from 3. This is the eighteenth consecutive year rabid bats have been taken in western Montana. Up to now we have identified rabies in a total of 51 bats. One dog among 8 other animals examined for rabies was infected.

"Distemperoid", a strain of canine distemper virus innocuous to dogs but lethal for skunks is being considered as a possible means for control of rabies-infected skunk populations. For purposes of obtaining information on natural occurrence of distemper among skunks, neutralization tests were done on the serum of skunks from a rabies enzootic area in North Dakota. One of 87 serums reacted with significant titer.

Significance to biomedical research and the program of the Institute:

We have demonstrated that abortive rabies infection can be experimentally produced in mice, dogs and cats. Criteria for proven recovery in these animals were used to demonstrate absence of nonfatal rabies in a large group of dogs in an enzootic area. Presence of rabies antibodies for 90 weeks in CSF of experimentally infected and recovered dogs but their absence in vaccinated dogs provides a basis for identifying abortive rabies 2 years or longer after infection. Findings to date suggest that the blood/brain barrier is not breached to humoral antibody in experimental allergic encephalomyelitis (and presumably its counterpart, "vaccinal encephalitis"). Much confusion exists in the differential diagnosis of

abortive rabies and neuro-paralytic reaction to rabies vaccine. Antibodies in serum may be present in both conditions, but, according to recent findings, CSF antibodies would signify rabies encephalitis only.

There is also much confusion regarding differential diagnosis of specific neuro-paralytic reactions to rabies vaccine and Landry-Guillain-Barre syndromes which resemble postvaccinal paralysis, but may, or may not, be etiologically related. We believe that CSF serology can be utilized to bring order and understanding to a chaotic field.

While increased ambient temperature apparently inhibits rabies infection, it has not increased survival after onset of disease. We cannot see useful application of these findings to medical practice but they may be helpful in understanding the epizoology of rabies.

We are encouraged by the significant increase in survival stimulating HSF of H. pertussis, administered simultaneously with rabies virus. Whether this has some application to human and veterinary medicine will depend upon results of current experiments in animals other than mice. Our findings may afford additional information on some of the mechanisms of action of HSF.

Untoward reactions to rabies vaccines of all kinds are grossly underreported. Sequential injections of a vaccine appear to give more unfavorable reactions than do single doses, and in most cases the menstruum rather than virus antigen is responsible. Successive inoculations stimulate undesirable antibodies to menstrual antigen in addition to the desired response to the specific virus antigen. It would be advantageous to have a choice of vaccines available in both human and veterinary medicine for (1) persons (animals) allergic to one menstruum on initial inoculation, (2) "Pasteur series" treatment after rabies exposure in man, (3) booster inoculations for animals and persons in high-risk areas, and (4) persons who develop adverse reactions while under treatment. Present policy is to standardize production and licensing of vaccines with limited choice. We believe that our current experiments will demonstrate the advisability of diversified options and use.

Outcome of current studies on water-borne tularemia will depend to some extent on continued natural contamination of the stream under study. Our investigations have provided much circumstantial evidence that contamination of surface waters during epizootics is derived from infected rodents. Present efforts are directed toward demonstration of a similar relationship between intermittent contamination and enzootic tularemia, a much more difficult project. Occasional human infection from contaminated water may be prevented if we can show the source of the organism. Corollary studies showed that tularemic nephritis is derived from hematogenous rather than retrograde infection. The role of hypersensitivity to F. tularensis in the pathogenesis of nephritis is obvious from these studies.

Proposed course of project:

No major changes in direction are planned; future studies will depend on the results of current and planned experiments. We will continue surveillance of rabies and our diagnostic service to physicians, veterinarians, university faculty, other government agencies, etc.

Honors and Awards:

Dr. J. F. Bell

Speaker at seminar of the Epidemiology Section, CDC, Atlanta, on Current Developments in Abortive Rabies, March 14, 1972.

Appointed Adjunct Professor, Departments of Microbiology and Zoology, University of Montana, Missoula.

Publications:

Stoenner, H. G.: Differential diagnosis of leptospirosis in man. Rocky Mountain Med. J. 68: 25-29, 1971.

Bell, J. F., Gonzalez, M. A., Diaz, A. M., and Moore, G. J.: Nonfatal rabies in dogs: experimental studies and results of a survey. Amer. J. Vet. Res. 32: 2049-2058, 1971.

Bell, J. F., Sancho, M. I., Dias, A. M., and Moore, G. J.: Nonfatal rabies in an enzootic area: results of a survey and evaluation of techniques. Amer. J. Epidem. 95: 190-198, 1972.

In press:

Bell, J. F.: Latency and abortive rabies. In Baer, G. M. (Ed.): The Natural History of Rabies. New York, Academic Press.

Owen, C. R.: Genus Francisella. Section in Bergey's Manual of Determinative Bacteriology (ed. 8). Baltimore, Williams & Wilkins.

Serial No. NIAID-174
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Systematics and vector relationships of certain parasitic arthropods

Previous Serial Number: Same

Principal Investigator: J. M. Brennan

Other Investigators: C. M. Clifford, E. K. Jones, J. E. Keirans, R. Parker (NIH Postdoctoral Fellow), and C. E. Yunker

Cooperating Units:
Mr. A. B. Amerson, Jr., University of Kansas, Lawrence
Dr. M. Chitwood, National Center for Primate Biology, Davis, Calif.
Dr. A. Fain, Institute of Tropical Medicine, Antwerp, Belgium
Dr. C. O. Handley, Smithsonian Institution
Dr. S. Herrin, University of Southern Georgia, Statesboro
Dr. H. Hoogstraal, NAMRU-3, Cairo, Egypt
Dr. K. Johnson, MARU, Canal Zone
Dr. J. Jones, Texas Technical University, Lubbock
Dr. M. Khalil, Research Institute, Medical Entomology, Dokki, Egypt
Dr. G. W. Krantz, Oregon State University, Corvallis
Dr. R. B. Loomis, California State College, Long Beach
Dr. F. Lukoschus, Katholieke Universiteit, Nijmegen, The Netherlands
Mr. C. MacFarland, University of Wisconsin, Madison
Dr. C. Marinkelle, Universidad de los Andes, Bogota, Colombia
Dr. F. J. Radovsky, Bernice P. Bishop Museum, Honolulu, Hawaii
Mr. J. T. Reed, Brigham Young University, Provo, Utah
Dr. V. Rupes, Czechoslovakian Academy of Sciences, Prague
Mr. R. Saunders, Brigham Young University, Provo
Dr. D. Sonenshine, Old Dominion University, Norfolk, Virginia
Dr. V. J. Tipton, Brigham Young University, Provo

Dr. H. Trapido, Tulane University, Baton Rouge, La.
Dr. P. H. Vercammen-Grandjean, George Williams Hooper Foundation, San Francisco, Calif.
Dr. N. Wilson, University of Northern Iowa, Cedar Falls

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	5 9/12			5 9/12
Professional:	3 5/12			3 5/12
Other:	2 4/12			2 4/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 03-005-1	C. M. Clifford H. G. Stoenner	NAMRU-3 Cairo, Egypt	H. Hoogstraal	$29,200

Project Description:

Objectives:

Taxonomy and colonization of parasitic arthropods of known and potential medical importance.

Methods employed:

Standard, adapted, and conceived techniques for collecting, handling, rearing and colonizing parasitic arthropods and their preparation for taxonomic study. Collaboration with appropriate sections at RML and other agencies and institutions. Identification and clarification of systematic problems, principally of ticks and parasitic mites.

Major findings:

1. Ticks (Clifford, Keirans, and Jones). Transfer of the national tick collection from the Smithsonian Institution to RML has been completed. Transfer of Dr. Hoogstraal's tick collection and library is continuing.

Identifications of ticks, world-wide, were provided as requested to numerous agencies, schools and research institutions.

The collaborative projects on ticks of Venezuela and the systematic revision of Ixodes subgenera have recently been completed. A new collaborative project with NAMRU-3 and the University of Wisconsin on ticks of the Galapagos Islands is in progress.

Dr. Keirans has been named co-project officer with Drs. Clifford and Stoenner of PL-480 project 03-005-1.

2. Parasitic mites (Yunker and Parker).

a. Some 1500 collections of dermanyssoid mites of Venezuela have been mounted and all but 300 identified. All preparative work and a descriptive manuscript have been completed and other manuscripts are in preparation. Developing filaria larvae, subfamily Dipetaloneminae, were recognized in a mite parasitic on phyllostomatid bats. This group of filaria, which includes causal agents of human filariasis, is known from bats in many parts of the world, but vectors are unknown. The proven ability of parasitic mites and ticks to transmit certain species of dipetalonemine worms to other vertebrates, together with evidence that the larvae seen here had undergone development in the mite, suggests that mites are vectors of bat filariae. This finding has been prepared for publication. Collaborators: Tipton, Saunders, Radovsky, Herrin, Chitwood, Fain.

b. Approximately 225 collections of macronyssid mites of Surinam have been identified and about 25 remain to be studied. Collaborators: Lukoschus, Radovsky, Krantz.

c. An innovative study, departing from the classical approach to taxonomy, was started, in collaboration with Dr. Hoogstraal: "Biochemical taxonomy of ticks with special reference to the Haemaphysalis longicornis complex". In this, hemolymph proteins and isoenzymes from various genera, species and infraspecific populations of ticks are analyzed by polyacrilamide-disc electrophoresis. Differences in electrophoretic mobility and heat lability are used to determine relatedness among forms. So far, we found that hemolymph proteins from Dermacentor andersoni, D. variabilis and D. albipictus showed similar patterns readily distinguishable from those from H. leporispalustris. Significant differences in electrophoretic mobilities of lactate and succinate dehydrogenase isoenzymes from the three Dermacentor species and H. leporispalustris were seen. A colony of parthenogenetic, triploid H. longicornis was established, but the study was hampered by loss of our colony of H. lagrangei.

Of special significance to this project is the fact that molecules under single gene control, such as isoenzymes, can serve as stable genetic

markers and thus provide evidence of phylogeny and speciation not obtainable through conventional taxonomic means. The significance of this study to biomedical research stems from the ability of members of the difficult H. longicornis complex to transmit a number of disease agents infectious to man or domestic animals (e.g., Russian spring-summer encephalitis virus, Q fever rickettsia, various theilerias).

d. Other studies completed or in progress and collaborators are: Central American macronyssids (Jones); Colombian bat parasites (Trapido); Colombian macronyssids (Marinkelle); Bolivian macronyssids (Johnson); hypopid mites (Wilson, Rupes); Egyptian and Indonesian mites (Hoogstraal, Khalil); endoparasitic chiggers (Vercammen-Grandjean).

3. Chiggers (Brennan). Assisted by Jack Reed, Brigham Young University, identifications of the large Venezuelan collection, about 20,000 slides, are practically completed. About 50 undescribed species have been recognized, half of these in genus Eutrombicula, the medically important group of pest chiggers. Pertinent to this study, 2 reports have been published, 2 are in press, and others (including a comprehensive study of Eutrombicula) are in preparation.

As part of a continuing study of Neotropical chiggers, identifications of Surinam material are still in progress with possibly 600 slides to be examined. Two reports of this work have been published. Collaborator: Lukoschus.

The study of Central Pacific chiggers in conjunction with the Smithsonian Pacific Ocean Biological Survey Project was completed and a second report published. Collaborator: Amerson.

Identifications were made for various organizations and individuals as requested.

4. Published new species and genera (in parentheses) according to faunal regions are:

	Oriental	Australian	Neotropical
Ticks	2		8
Chiggers		6	2(1)

5. Tick, mite, and mosquito colonies. Laboratory stock colonies are used for transmission studies and are supplied to investigators throughout the world. The RML insectary provided nearly 50,000 ticks of 9 species for research needs here and elsewhere.

Eggs and larvae of Culex tritaeniorhynchus were obtained from WRAIR and this species is now under colonization. Studies are under way to determine whether this common vector of Japanese B encephalitis virus can be infected with and transmit Tuleniy virus.

Currently, 27 tick, 3 mite and 3 mosquito species are colonized.

Significance to bio-medical research and the program of the Institute:

As in last annual report, where arthropods are concerned in human disease, their systematic studies are basic and a must to any and all research issuing therefrom. The taxonomically and biologically trained personnel of this section, in addition to providing numerous identifications, are frequently consulted for information involving protection from and control of medically important arthropods, and are even appealed to for aid in solving problems of entomophobia. Over and above basic research, various services are provided. The arthropod colonies are used in arthropod tissue culture, transmission studies, establishing sub-colonies, etc.

Proposed course of project:

Continued investigations of parasitic arthropod systematics and biology, and provision of services, with emphasis on ticks, mites, and mosquitoes.

Publications:

Philip, C. B. and Coscaron, S.: New Neotropical Tabanidae. II. Three primitive undescribed pangoniine-like flies of unusual interest from Chile. Papeis Avulsos de Zoologia 23: 127-136, 1971.

Rupes, V., Yunker, C. E., and Wilson, N.: Zibethacarus, n. gen. and three new species of Dermacarus (Acari: Labidophoridae). J. Med. Entom. 8: 17-22, 1971.

Brennan, J. M.: Endoparasitic chiggers: VI. Neoschoengastia esorhina n. sp. (Acarina: Trombiculidae), a second intranasal chigger of birds. J. Parasit. 57: 666-667, 1971.

Brennan, J. M. and Lukoschus, F.: Parasitic mites of Surinam. VIII. A new genus and species of chiggers, Fauranius atecmartus, and additional records of species (Acarina: Trombiculidae). Bull. Southern Calif. Acad. Sci. 70: 42-45, 1971.

Radovsky, F. J. and Yunker, C. E.: Xenarthronyssus furmani, n. gen., n. sp., (Acarina: Dasyponyssidae), parasites of armadillos, with two subspecies. J. Med. Entom. 8: 135-142, 1971.

Stout, I. J., Clifford, C. M., Keirans, J. E., and Portman, R. W.: Dermacentor variabilis (Say) (Acarina: Ixodidae) established in southeastern Washington and northern Idaho. J. Med. Entom. 8: 143-147, 1971.

Keirans, J. E., Clifford, C. M., and Capriles, J. M.: Argas (Argas) dulus, new species (Ixodoidea: Argasidae), from nests of the palm chat Dulus dominicus in the Dominican Republic. Ann. Entom. Soc. Amer. 64: 1410-1413, 1971.

Clifford, C. M., Hoogstraal, H., and Kohls, G. M.: Ixodes hyatti, n. sp., and I. shahi, n. sp. (Acarina: Ixodidae), parasites of pikas (Lagomorpha: Ochotonidae) in the Himalayas of Nepal and West Pakistan. J. Med. Entom. 8: 430-438, 1971.

Clifford, C. M., Keirans, J. E., and Hoogstraal, H.: Ixodes (Ixodes) nuttallianus Schulze: redescription of female, description of male, and hosts and ecology in Nepal (Acarina: Ixodidae). J. Med. Entom. 8: 439-442, 1971.

Fay, R. W. and Keirans, J. E.: Production of Aedes mediovittatus (Coquillett) and its response to temperature and food conditions. Mosquito News 31: 488-491, 1971.

Brennan, J. M. and Amerson, A. B.: Six new species and additional records of chiggers from the Central Pacific (Acarina: Trombiculidae). J. Parasit. 57: 1311-1317, 1971.

Brennan, J. M.: Anomalaspis ambiguus Brennan, 1952 redescribed and the questionable association of South American species with Pseudoschoengastia (Acarina: Trombiculidae). J. Med. Ent. 8: 602-604, 1971.

Brennan, J. M.: A new genus and two new species of Venezuelan chiggers with eyes in the scutum (Acarina: Trombiculidae). J. Med. Ent. 9: 16-18, 1972.

In press:

Brennan, J. M. and Reed, J. T.: Loomisia, new genus with descriptions of three new Venezuelan species (Acarina: Trombiculidae). J. Parasit.

Brennan, J. M, and Reed, J. T.: Two new Venezuelan chiggers of the genus Polylopadium (Acarina: Trombiculidae). J. Med. Ent.

Keirans, J. E. and Jones, E. K.: Description of the immature stages of Ixodes (I.) sinaloa Kohls and Clifford (Acarina: Ixodidae), from rodents in Mexico and Nicaragua. Acarologia

Keirans, J. E., Backhurst, G. C., and Clifford, C. M.: Description of the male of Ixodes walkerae with notes on the female and description of the males of Ixodes daveyi and Ixodes thomasae (Acarina: Ixodidae). Rev. Zool. Bot. Afr.

Keirans, J. E.: Redescription of Amblyomma fulvum Neumann, 1899 (Acarina: Ixodidae), a parasite of the giant anaconda in northern South America. J. Med. Entom.

Yunker, C. E.: Ch. 15: Acarina other than ticks. In Flynn, R. J. (Ed.): Diseases of Laboratory Animals. Iowa State Univ. Press, Ames.

Serial No. NIAID-175
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Allergy, its mechanisms and role in disease

Previous Serial Number: Same

Principal Investigators: J. J. Munoz, R. K. Bergman, and J. E. Coe

Other Investigators: W. J. Hadlow, R. C. Kennedy, G. Lavelle, D. Lodmell, and R. E. Race

Cooperating Units: Drs. J. Jutila and N. Reed, MSU, Bozeman, Montana
Dr. Hans Spiegelberg, Scripps Institute, LaJolla, Calif.
Dr. E. Leonard, NCI, NIH, Bethesda, Maryland
Drs. Peters and Johnson, MARU, Canal Zone
Dr. Bill Hoyer, Carnegie Institution, Washington, D.C.
Mr. J. D. Converse, University of Montana, Missoula

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	8 3/12		1 3/12	9 6/12
Professional:	2 2/12		1 3/12	3 5/12
Other:	6 1/12			6 1/12

Project Description:

Objectives:

1) To study the fundamental bases of hypersensitivity and its role in disease, 2) to learn more about the function of immunoglobulin classes, and 3) to investigate the mechanisms of infection in slow virus infections.

Methods employed:

Typical immunological, biochemical, biophysical and serological procedures are used.

Major findings:

1. Delayed hypersensitivity in mice (Munoz). Arthus reactions in mice can be demonstrated in the skin, providing antibody titers to the antigen are high, but we have never seen true delayed hypersensitivity (DH) in the skin of mice. Crowle has observed that delayed reactions can be produced in mice with various antigens, and particularly with methylated bovine serum albumin (MBSA) dissolved in phosphate buffered saline. With this antigen, mice develop little or no immediate reactions and a marked delayed (24 to 48 hr) response. We reinvestigated this phenomenon and obtained the following findings: 1) Mice sensitized with 2 injections of MBSA given subcutaneously 7 days apart responded with delayed skin reactions in the absence of visible 4 hr reactions. These reactions could be observed better in the footpad than in the skin. 2) When the sensitizing dose of antigen was dissolved in M/15 phosphate buffered saline instead of plain saline DH was more pronounced (this was also observed by Crowle). 3) Retesting the same mice 14 days after the first skin test produced strong immediate reactions (4 hr) which persisted for 48 hr or longer. 4) Macrophage migration inhibition (MIF) tests performed with peritoneal macrophages of mice known to have delayed skin or footpad reactions were invariably negative, regardless of whether they were sensitized with MBSA, human serum albumin, or mycobacterial oil-cell-wall vaccine (PPD used as test antigen). 5) Passive transfer of immediate sensitivity to MBSA was easily accomplished with anti-MBSA prepared in mice. 6) The gross appearance of the delayed and the immediate reactions was not obviously different and both had little erythema. 7) The footpad test was superior to the skin test for detecting immediate or delayed responses in the mouse.

2. Studies on delayed hypersensitivity in mink (Munoz, Lodmell, Race, and Hadlow). This study was undertaken to establish whether mink were capable of developing DH as measured by the skin, footpad, or macrophage migration inhibition tests.

Pastel mink were immunized with either hen's egg albumin (Ea), keyhole limpet hemocyanin (KLH), insulin (IN) or Mycobacterium tuberculosis (BCG) cell walls in oil (CW). Of these antigens only the CW was capable of stimulating DH in pastel mink as measured by the skin and by MIF tests. Although skin tests for DH and MIF tests were negative in mink immunized with Ea, KLH or IN, antibodies to Ea and KLH were easily detected in serums of immunized mink. Antibodies to PPD were also detected in mink immunized with CW. These antibodies did not correlate with the inhibition of macrophage migration.

3. Antibody response in thymusless mice (Munoz). In collaboration with Drs. Jutila and Reed of MSU, we have studied the antibody response of thymusless (nude) mice to determine if nude mice made γE-like antibody after inoculation with an antigen mixed with an adjuvant such as Bordetella pertussis extracts (SE). Nude mice failed to develop antibodies to protein

antigens. These mice, after inoculation with Ea, KLH, or SE, did not produce 2-hr PCA antibody ($7S_{\gamma 1}$), 72-hr PCA antibody (γE-like), nor hemagglutinating antibodies (most immunoglobulins including γM and possibly γA). Nude mice responded to immunization with Escherichia coli endotoxin with the possible production of γM antibodies; the thymus containing litter mates responded as well as or better than CFW mice with the production of $7S_{\gamma 1}$ and γE-like antibodies, and perhaps also with $7S_{\gamma 2}$, γM and γA. By Schultz-Dale test we showed that the uteri of nude mice previously immunized to different antigens failed to contract in presence of specific antigen, although uteri of litter mates and CFW mice showed specific sensitivity to these antigens. All uteri (from nude, litter mates or CFW mice) contracted in the presence of serotonin. E. coli endotoxin, although stimulating antibody response, did not sensitize the uteri of nudes, litter mates, or CFW mice.

4. Studies on peromyscus (Coe). The $7S_{\gamma 1}$ globulins of peromyscus were found to differ in their electrophoretic mobility. Three electrophoretic variants (phenotypes) were defined: a homogeneous fast, homogeneous slow or heterogeneous fast-slow. This $7S_{\gamma 1}$ phenotype was genetically determined and was due to structural differences on the $7S_{\gamma 1}$ Fc piece (H chain). By breeding experiments, this polymorphism was shown to be the result of at least two allelic $7S_{\gamma 1}$ H chain genes which produced either the homozygous fast or slow phenotype or the heterozygous fast-slow phenotype. Allotypy of immunoglobulins expressed by electrophoretic variation is quite rare—the only other example being the $7S_{\gamma 1}$ globulins in Mus musculus, a distant relative of peromyscus. This suggests that the genetic event responsible for this $7S_{\gamma 1}$ polymorphism occurred in a common ancestor and therefore a comparable $7S_{\gamma 1}$ polymorphism should be present in other rodents.

The genus peromyscus is probably the most heterogeneous group of rodents in North America. This heterogeneity provides a fertile field for studies on evolution, and in collaboration with Dr. Bill Hoyer (Carnegie Institution, Washington, D.C.) studies on DNA homology and satellite DNA are being conducted.

5. Studies on mink (Coe and Hadlow). Mink "monoclonal" immunoglobulins (Ig) are being isolated by electrophoretic and chromatographic techniques. Studies so far substantiate the homogeneity of mink "monoclonal" Ig, i.e., they have idiotypic determinants and only one L chain type. Further studies on the homogeneity of the light and heavy chains of mink "monoclonal" proteins will substantiate whether or not these proteins are of monoclonal origin.

In collaboration with Dr. Hadlow, studies on the effect of X-ray on Aleutian disease (AD) have been initiated. So far these studies have shown that 1) mink are X-ray susceptible, i.e., 300 (or more) r total body irradiation was lethal (under adverse winter environmental conditions), and 2) 100 r given before AD injection did not appear to alter the course of disease.

6. Studies with bullfrog antibodies (Coe). Previous studies at RML have shown that bullfrog antibody has a specificity which can be distinctly different from that of a mammalian antibody to the same protein. This unique immunological perspective, probably a result of the phylogenetic distance of amphibian from mammals, is being used in several different ways. In collaboration with Dr. Hans Spiegelberg (Scripps Institute, LaJolla, Calif.) bullfrog antiserums have been produced against various human IgA and IgD myeloma proteins in an attempt to discern new subclasses. The results indicate that bullfrog antiserums are especially useful for these studies because 1) subclass antigens are readily detectable in most antiserums (in contrast to mammalian antiserums), 2) idiotypic antibodies are much less prevalent than that found in mammalian antiserums. Furthermore, little antibody to the L chain antigens is produced, although the tertiary configuration imposed by λ and κ light chains appears to have a significant influence on the antigenicity of the molecule. In collaboration with Dr. E. Leonard (NCI, NIH) we have been attempting to produce an antibody to human protein which binds to myocardium. Studies on this interesting protein have been frustrating because of its apparent nonantigenicity in other mammals. Preliminary results indicate that an antibody is being produced in bullfrogs and further studies by indirect fluorescent microscopy will define the specificity of this antibody.

7. Studies on Calomys callosus (Coe). In collaboration with Drs. Peters and Johnson (MARU), we have defined 3 distinct immunoglobulins in the rodent (Calomys callosus) which is an important reservoir of Machupo virus. Antiserums specific for a $7S\gamma 1$, $7S\gamma 2$ and γM globulins of Calomys have been prepared and will be utilized to define the immunoglobulins involved in the antibody response to Machupo virus. These studies may help explain why a carrier state of Machupo virus exists in some Calomys.

8. Studies on immunoglobulin (Ig) metabolism in the Syrian hamster (Converse). Previous observations in this laboratory have indicated that major alterations in the serum Ig concentration occur after immunization. Of particular interest was the finding that mineral oil emulsions, which did not even contain antigen, could induce comparable alterations of serum Ig levels; that is, when incomplete Freunds adjuvant (IFA) without added antigen was injected intraperitoneally, serum IgM concentrations increased in some animals up to 10 fold greater than that of the normal. The metabolism of IgM was studied using radiolabeled IgM and the results indicate that IFA (without added antigen) causes increased serum IgM levels by increasing the IgM synthetic rate and not by decreasing IgM catabolism. For instance, preliminary calculations indicate that IFA increases the IgM synthetic rates (per 100 gm body weight) from 3 mg to 10 to 11 mg per day. The stimulation of IgM synthesis was not detected when complete Freunds adjuvant was used; this apparent inhibition of the IFA effect on IgM by mycobacteria will be studied further. The biological half life of IgM in normal hamsters (or in hamsters with increased IgM serum concentration) was 24 hrs and the biological half lives of $7S\gamma 2$ and IgA were 3 days (2.5 to 3.5) and 20 hrs respectively.

Significance to biomedical research and the program of the Institute:

Delayed skin hypersensitivity reactions in mice have always been questionable due to the presence of circulating antibodies and immediate skin sensitivity which complicates the readings of delayed reactions. With MBSA (a strongly positively charged antigen) delayed skin or footpad reactions were obtained. The mechanism by which MBSA produces this delayed response is not yet clear, but it is obvious that the methyl groups attached to the BSA are extremely important. This points to a role of electrical charge on the antigen as an important factor on the demonstration of delayed reactions in the skin of mice. The role of antibodies on this reaction has not been excluded. The macrophages of mice sensitized to MBSA are not inhibited from migrating in vitro by this antigen. These studies may eventually explain the mechanism of induction of DH in mice and in turn may shed some light on DH in general.

Our studies on DH in mink were undertaken with the aim of studying the effect of AD on the development of DH in these animals. It is already known that AD produces a marked inhibitory effect on antibody production to antigens given at the height of infection. It would be interesting to know if the DH response of mink is also affected by AD.

The study of antibody responses of thymusless mice, especially with reference to the effect of the adjuvant action of B. pertussis extracts, is of significance because it showed that, with protein antigens, the thymus is needed for the production of γE-like antibody as well as for the production of antibodies belonging to other globulin classes. It further confirmed that the thymus is not necessary for the production of antibodies (probably γM) to E. coli endotoxin. These studies shed some light on the role of thymus on antibody production and hypersensitivity, as reflected by results of Schultz-Dale tests.

The studies on mink immunoglobulins may give some insight into the mechanism of AD in mink. With this aim in mind the studies of X-radiation of mink are also of importance because they would indicate whether the immune response operates during AD virus infection. The utilization of the bullfrog for the production of antibodies to antigens on antigenic determinant groups that do not stimulate antibodies in mammalian species has been of value in various problems.

The studies on the heterogeneity of 7Sγ1 immunoglobulins of peromyscus furnish a tool to investigate the evolution of these immunoglobulins in the very heterogeneous peromyscus genus.

Proposed course of project:

This project will continue to emphasize the studies of slow virus infections. In addition, other aspects of delayed hypersensitivity in mice will be studied and the study of immunoglobulins in various species and the function of the various immunoglobulin classes will be continued.

Honors and Awards:

Dr. J. J. Munoz

Continued as Associate Editor of the Journal of Immunology.

Courtesy appointment as Faculty Affiliate, University of Montana.

Serve as member of the Council for the Mid-Winter Immunologist Conference.

Dr. J. E. Coe

Courtesy appointment as Faculty Affiliate, University of Montana.

Publications:

Haakenstad, A. O. and Coe, J. E.: The immune response in the hamster. IV. Studies on IgA. J. Immun. 106: 1026-1034, 1971.

Coe, J., Peel, L., and Smith, R. F.: The immune response in the hamster. V. Biological activities of $7S_{\gamma 1}$ and $7S_{\gamma 2}$ globulins. J. Immun. 107: 76-82, 1971.

Coe, J. E.: The immune response in the hamster. III. Selective induction of concomitant antibody formation and unresponsiveness in the $7S_{\gamma 1}$- and $7S_{\gamma 2}$-globulins with soluble hen egg albumin. Immunology 21: 175-191, 1971.

Munoz, J.: Double diffusion in plates. In Williams, C. A. and Chase, M. W. (Eds.): Methods in Immunology and Immunochemistry. New York, Academic Press, Inc., 1971, vol. 3, pp. 146-160.

Coe, J. E. and Hadlow, W. J.: Studies on immunoglobulins of mink: definition of IgG, IgA and IgM. J. Immun. 108: 530-537, 1972.

Publications (Cont'd)

Coe, J. E.: Studies on immunoglobulins of mink: definition of two populations of light chains. Immunochemistry 9: 147-151, 1972.

Coe, J. E.: Studies on IgA of the guinea pig. Immunology 22: 333-345, 1972.

In press:

Coe, J. E.: Immune response in the turtle (Chrysemys picta). Immunology

Coe, J. E.: A genetic polymorphism of peromyscus 7Sγ1 globulins: detection by differences in electrophoretic mobility. J. Immun.

Coe, J. E. and Takemoto, K. K.: Immune response in the hamster. VI. Antibody response in polyoma oncogenesis. J. Nat. Cancer Inst.

Serial No. NIAID-177
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Immune prophylaxis of mycobacterial infections

Previous Serial Number: Same

Principal Investigators: R. L. Anacker and E. Ribi

Other Investigators: W. D. Bickel, W. R. Brown, G. Goode, R. H. List, T. J. Meyer, and R. F. Smith

Cooperating Units: Dr. W. R. Barclay, AMA, Chicago
Dr. W. Brehmer, Rober Koch Institute, Berlin, Germany
Dr. S. C. Harris, Stanford Medical Center, Stanford
Dr. R. L. Hunter, University of Chicago
Dr. M. Kato, Research Institute of Tuberculosis, Osaka, Japan
Dr. J. Matsumoto, Research Institute of Tuberculosis, Osaka, Japan
Dr. H. Wolochow, Naval Biomedical Research Lab, Oakland
Dr. K. Yamamoto, Hokkaido University, Sapporo, Japan

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	11 8/12		1/12	11 9/12
Professional:	5 10/12		1/12	5 11/12
Other:	5 10/12			5 10/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
N000-14-69-A0200	E. Ribi	Nav Biomed Lab Oakland, Calif	H. Wolochow	$24,201

Project Description:

Objectives:

To develop an effective nonliving antituberculosis vaccine from purified fractions of mycobacteria, to study the nature of the host responses to these fractions, and to determine the relationship of delayed hypersensitivity to immunity.

Methods employed:

A variety of physical, chemical and immunologic techniques was used to prepare and characterize cell walls (CW) and other fractions of several mycobacterial species and strains and to assay the response of experimental animals to these mycobacterial fractions. Protective potency of these preparations was determined in mice, monkeys, and guinea pigs challenged by aerosol with Mycobacterium tuberculosis H37Rv.

Major findings:

1. Vaccine trials in monkeys. Evaluation of the BCG CW vaccine in rhesus monkeys has been continued in collaboration with staff at the Naval Biomedical Research Laboratory. Protection against airborne infection afforded by M. bovis Bovinus I CW given intravenously was compared with that provided by viable BCG also given intravenously 2, 6, and 12 months postimmunization. Examination of organs from monkeys challenged at the longest interval has not been finished, so the results are necessarily incomplete.

Sensitivity of monkeys to PPD given intrapalpebrally depended upon the interval between immunization and skin testing. Only 7 of 34 animals responded minimally to 250 tuberculin units (TU) PPD at 2 months after vaccination with CW, but 14 of 23 exhibited weak to strong reactions after 3 months. This late development of sensitivity was not demonstrated in earlier experiments because of their limited duration. Only 5 of 12 monkeys gave minimal reactions after 12 months. Tuberculin sensitivity of BCG-vaccinated monkeys roughly varied in a similar manner. Six of 10 animals gave weak to strong reactions to 250 TU after 6 weeks and 8 of 10 after 2 months. After 12 months only 4 of 12 gave even a weak response.

Some evidence on the duration of the granulomatous response induced by CW, and to a lesser extent by BCG, has been obtained in this experiment. All but 1 of the 10 CW-vaccinated animals autopsied 2 or 4 1/2 months postvaccination had slight to moderate degrees of granuloma formation in their lungs. Most of the BCG-immunized animals had evidence of lymphocytic infiltration in one or more organs at these same intervals. Both CW-vaccinated monkeys sacrificed at 6 months had low-grade pulmonary granulomatous reactions, and 3 of 8 lungs obtained 2 1/2 months later appeared

normal. Lungs of all 10 BCG-vaccinated monkeys examined at these two later intervals were also normal.

A preliminary scoring of the extent of tuberculous disease in animals autopsied 10 weeks after challenge infection, as determined by macroscopic and microscopic examination of tissue, is presented in the following table. The rather high level of resistance found 2 months after immunization decreased in both vaccinated groups by 6 months. However, the drop in resistance was much greater for the CW-vaccinated animals than for the BCG-vaccinated monkeys.

Mean score of tuberculous disease in CW- or BCG-vaccinated rhesus monkeys infected 2 or 6 months postimmunization

	Interval between vaccination and challenge	
	2 mos	6 mos
M. bovis Bovinus I cell walls	16.3	42.5
BCG	0	12.5
Controls	61.3	53.8

0 = no disease; 100 = maximum disease

2. Tuberculoimmunity and delayed hypersensitivity. A second experiment to determine the ability of BCG CW and viable BCG to prolong survival of guinea pigs infected with H37Rv by the airborne route was completed. Results of the first trial indicated the superiority of the subcutaneous route over the intravenous route for this species, a result exactly opposite to that found for mice and rhesus monkeys. The results of our most recent experiment confirmed the earlier finding. That the "long-term survival" tests with guinea pigs agree well with those of the "short-term sacrifice" tests performed several years ago is significant. In addition, a dose of 0.2 mg of CW given intravenously was found to protect guinea pigs significantly better than did a dose 5 times larger given by the same route. Such a result is consistent with the hypothesis that a dose of CW greater than the optimum dose would be detrimental to protection because of the high level of delayed hypersensitivity engendered.

In collaboration with Dr. R. L. Hunter an attempt was made to stimulate tuberculoimmunity in mice with a complex of acid-inactivated CW, a conjugate of PPD and lauric anhydride, and a lipid extract. In earlier experiments, a combination of this CW and the lipid only was not protective, but the addition of a crude "protein" extract to the first 2 components provided an immunogenic complex. However, in this instance lauryl-PPD had

no beneficial effect. Either PPD is not an essential ingredient of a potent vaccine, or for technical reasons the PPD was not complexed in an appropriate way with the other constituents.

The study of the role of the sensitized mouse lymphocyte in resistance to tuberculosis was continued. It was learned earlier that syngeneic recipients of lymphoid cells from immune donor mice were no more resistant to airborne infection with H37Rv than were normal control mice unless the recipient mice also were given a booster dose of oil-treated CW. Since the booster dose sometimes afforded a significant level of resistance to the control mice, the effect of boosting with nonprotective mycobacterial preparations was recently determined. Neither CW without oil, NaOH-inactivated CW, nor PPD was consistently effective in this experimental model.

In an effort to find a method to protect mice against aerosol challenge with BCG CW free of mineral oil, we tested the ability of alum-aggregated CW to stimulate resistance when presented by several routes and in multiple doses, but none of these modifications in procedure was protective. Histologic examination of the lungs showed only very small granulomas around occluded vessels in intravenously vaccinated animals. Control lungs from animals which received oil-treated-CW intravenously showed the usual massive granulomatous response.

Tuberculin shock, a well known expression of systemic sensitivity to tuberculin in a variety of experimental animals, has been recognized for many years, but the exact mechanism of this response is poorly understood. A study of this phenomenon in C57B1 mice was recently begun in this laboratory. These mice vaccinated intravenously with oil-treated BCG CW were found to be uniformly and fatally shocked by a 1-mg dose of PPD given by the intravenous route. The most remarkable histopathological finding thus far obtained is the presence of large numbers of lymphocytes in pulmonary vessels during the first 12 hours after challenge with PPD. Other studies are in progress.

To study the relationship between delayed hypersensitivity and acquired resistance, mice were given multiple intravenous injections of either BCG CW or PPD in saline for several weeks prior to vaccination with oil-treated CW. Resistance to airborne challenge and footpad sensitivity to PPD were greatly diminished in mice pretreated with CW but not with PPD. In addition, lungs of mice pretreated with CW before inoculation of oil-treated CW were significantly smaller than lungs of control mice given oil-treated CW only. These data indicate a relationship between immunity and delayed hypersensitivity and that mice can be made tolerant to a strong inducer of granuloma and delayed hypersensitivity by appropriate pretreatment.

3. Immunogenic and adjuvant activities of cell walls. Since, as reported previously, a combination of 2 nonprotective mycobacterial fractions, NaOH-inactivated CW and a lipid extract, was protective in mice challenged with H37Rv, it was suggested that the extract contained an adjuvant substance which enabled the mouse to respond in an effective manner to the immunogen which remained in the NaOH-treated CW. To test this hypothesis we compared the adjuvant activity of our mycobacterial combinations with that of untreated CW in a variety of experimental situations. CW enhanced antibody formation in mice to egg albumin but a mouse protective combination did not. Second, CW but not the combinations promoted delayed hypersensitivity to DNP-human serum albumin in guinea pigs. In contrast, the combination, but neither component alone, and untreated CW induced footpad sensitivity to PPD in mice. Finally, the combination was more effective than either component alone in inducing experimental allergic encephalitis in guinea pigs. These results are difficult to interpret at present but it is possible that different compounds are responsible for activity in different assay systems, and that no single "adjuvant" exists.

(No attempt is made to compare the above results with those obtained in guinea pigs inoculated with tumor cells and mycobacterial fractions (Project NIAID-179). Without additional data from this newer experimental system, any comparison made now would be highly speculative.)

4. Fractionation of mycobacteria. Studies to identify biologically active components in mycobacterial lipid extracts were continued. Development of pressure elution microparticulate gel chromatography (see Project NIAID-179) enabled us to accumulate purified components in quantities sufficient for experimentation.

One product selected for study was a cord factor sample prepared by Dr. Kato. Using the solvent system chloroform-methanol-water (85:15:1.5), the starting material was resolved into 3 components designated P_1, P_2, and P_3. The last two were found to be homogeneous when chromatographed with other solvent systems, whereas P_1, the component of lowest polarity, was heterogeneous. P_3 when combined with NaOH-inactivated BCG CW conferred resistance to mice against airborne challenge with H37Rv comparable to that given by BCG. P_1 afforded little protection and P_2 has not been tested. It is not known at present whether other constituents of mycobacteria also protect mice against infection with tubercle bacilli. Dr. Kato found P_3 to inhibit markedly mitochondrial respiration and phosphorlation and to possess a higher toxicity for mice than any cord factor sample he had tested in the past decade. P_1 and P_2 had toxicity of a lower order.

Cord factor was reported to be α-D-trehalose-6,6-dimycolate. Of the 3 bands isolated here, only P_3 showed the infrared absorption spectrum characteristic for trehalose. The spectrum for P_2 is similar to that for mycolic acid, and the spectrum for P_1 is quite different from those for P_2 and P_3.

The acetone-insoluble portion of a chloroform-methanol extract of BCG was also chosen for further purification because of its high protective activity when combined with NaOH-inactivated BCG CW. This product was chromatographed on LH20-Sephadex, and the cut which showed greatest protective activity (and which also contained a component resembling P3 described above) was resolved into 4 components by pressure elution chromatography. The protective potency of each of these 4 components when combined with inactivated CW is presently being determined.

Significance to biomedical research and the program of the Institute:

Data from these and earlier experiments suggest the possibility that the guinea pig may be a better animal in which to evaluate antituberculosis vaccines for human use than either the mouse or the rhesus monkey. In our hands, viable BCG and nonviable CW vaccines afford to the mouse and monkey minimal protection when given by the subcutaneous, intradermal, or intramuscular route and maximal protection when given by the intravenous route. In contrast, guinea pigs are protected significantly better by subcutaneously administered vaccine than by intravenously administered vaccine. Although similar comparisons are not available for man, evidence that BCG given intradermally to humans prevents the development of clinically recognized tuberculosis in most vaccinees has been well documented.

In addition, much recent evidence implicates the delayed hypersensitive state in resistance to infection with tubercle bacilli, and the guinea pig, like man, develops a high degree of hypersensitivity to tuberculoprotein. Certainly the mouse, and perhaps also the rhesus monkey, is a much poorer experimental animal than the guinea pig for the study of phenomena relating to delayed hypersensitivity. These considerations have led us to conclude that the guinea pig should be one of the animals utilized in our experiments directed towards the development of an effective, nonviable, nontoxic, antituberculosis vaccine.

Marked progress has been made during the past year in the fractionation of mycobacterial lipids by microparticulate bed chromatography. We believe that some of the more complex, immunologically significant, mycobacterial lipids have been isolated in the pure state for the first time. It will now be possible to determine the biological, chemical, and structural properties of these important components. Also, with this advance in technique, the possibility of creating an effective and nontoxic antituberculosis vaccine through combination of the essential, purified, protective components comes closer to realization.

Proposed course of project:

Several lines of investigation will be pursued in the coming year. The protective effect of purified components and of multiple doses of BCG CW given by peripheral routes to guinea pigs will be determined. Attempts to clarify the nature of the immune response will be made by determining

1) the tissue response and properties of cells of tolerant mice, 2) the relationship between distribution of antigen and organ immunity, 3) the conditions for effective passive transfer of resistance from immune to susceptible hosts, and 4) the effect of antimacrophage and antilymphocyte serum upon the vaccinated mouse. Study of tuberculin shock will continue in an effort to elucidate the means by which stimulated cells injure the host.

Much effort will be devoted to the isolation and biological and chemical characterization of the lipid components isolated by microparticulate bed chromatography. Among the analytical tools to be used are nuclear magnetic resonance analysis and infrared and mass spectroscopy.

Honors and Awards:

Dr. R. L. Anacker

Invited to present a paper at the Sixth Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in San Francisco, October 13-14, 1971.

Dr. E. Ribi

Continued as member, Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program.

Served as chairman of session at Sixth Annual Tuberculosis Conference sponsored by the Tuberculosis Panel of the U.S.-Japan Cooperative Medical Science Program in San Francisco, October 13-14, 1971.

Invited to participate at Conference on Immunization in Tuberculosis sponsored by Fogarty International Center, Bethesda, Md., Oct. 1971.

Invited with Drs. Milner and Parker to write a chapter on "Microparticulate Bed Chromatography Accelerated by Centrifugal Force and Pressure" for Methods of Biochemical Analysis.

Invited as guest lecturer at Workshop on Centrifugal Chromatography sponsored by Ivan Sorvall, Inc., Newark, N.J., June 1972.

Served as President of the Montana Tuberculosis and Respiratory Disease Memorial Foundation.

Served as reviewer for Science, Journal of Infectious Diseases, Infection and Immunity, Journal of Bacteriology, and NIH Allergy-Immunology Study Section.

Publications:

Ribi, E., Anacker, R. L., Barclay, W. R., Brehmer, W., Harris, S. C., Leif, W. R., and Simmons, J.: Efficacy of mycobacterial cell walls as a vaccine against airborne tuberculosis in the rhesus monkey. J. Infect. Dis. 123: 527-538, 1971.

Ribi, E.: Currents in tuberculosis research. J. Infect. Dis. 123: 562-565, 1971.

In press:

Anacker, R. L., Brehmer, W., Barclay, W. R., Leif, W. R., Ribi, E.: Simmons, J. H., and Smith, A. W.: Superiority of intravenously administered BCG and BCG cell walls in protecting rhesus monkeys (Macaca mulatta) against airborne tuberculosis. Z. Immunitatsforsch.

Anacker, R. L., Matsumoto, J., Ribi, E., Smith, R. F., and Yamamoto, K.: Partial purification of immunogenic material in lipid extracts of mycobacteria. J. Infect. Dis.

Serial No. NIAID-178
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Structure and biologic activity of endotoxins

Previous Serial Number: Same

Principal Investigators: K. C. Milner and E. Ribi

Other Investigators: W. D. Bickel and I. Azuma (Visiting Scientist, arriving in April 1972)

Cooperating Units: Dr. William R. Cross, U.S. Army Materials Research Agency, Watertown, Mass.
Dr. Lorraine Friedman, Tulane University
Dr. K. F. Meyer, University of California, San Francisco
Dr. J. A. Rudbach, University of Montana, Missoula

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	6 9/12		4/12	7 1/12
Professional:	1 7/12		4/12	1 11/12
Other:	5 2/12			5 2/12

Project Description:

Objectives:

To investigate the features of composition and structure of bacterial endotoxins (ET) required for expression of characteristic host reactivity.

Methods employed:

A combination of biophysical, chemical, and immunological approaches has been used.

Major findings:

1. Induced resistance to bacterial endotoxins (endotoxin tolerance) has been investigated further in order to complete the study for presentation at an international endotoxin conference this spring and publication in the proceedings of the conference (Journal of Infectious Diseases). The principal new material concerns a) extension of the study to effects other than pyrogenicity in rabbits, and b) examination of the ability of synthetic double-stranded RNA (poly I-poly C) to produce tolerance to its own endotoxin-like effects and reciprocal cross tolerance with endotoxin.

(a) As reported earlier a wholly nonspecific tolerance to pyrogenicity develops rapidly in response to a single small dose of endotoxin, then fades to be replaced by a specific late tolerance dependent upon antibodies to polysaccharide determinants. The latter type, not the former, can be transferred passively with serum of tolerant animals. Tolerance to the lethal effect of endotoxin does not follow this simple pattern. In rabbits, no significant resistance to lethal challenge developed within 24 hrs of preparation with a single dose of homologous or heterologous endotoxin, and only those vaccinated homologously were significantly resistant after 8 days. That mice become resistant to endotoxin after a series of preparatory inoculations is well known, but RML mice, at least, do not develop much resistance after a single prior exposure, regardless of dose or interval. They are also difficult to protect passively with hyperimmune sera; and there is some evidence that such protection may depend upon antibody to determinants other than the normal O antigen.

(b) Synthetic double-stranded polyriboinosinic-polyribocytidylic acid (poly I-poly C) is appreciably pyrogenic and lethal for laboratory animals although the reagents used to prepare it are not. A single preparative dose given 24 hrs before a standardized challenge with poly I-poly C produces tolerance to the pyretic effect, but the preparative dose must be 20 to 50 times more, in terms of activity, than would be required in an endotoxin system. Cross tolerance between endotoxin and poly I-poly C does not appear to occur.

2. Dr. William R. Cross, part of whose doctoral research was done with Dr. Milner as preceptor, now has the advanced facilities of the U.S. Army's "Molecular Characterization Laboratory" at his disposal. He sought our further collaboration in characterizing some of our preparations by physical methods other than the hydrodynamic techniques formerly employed. The preparation most studied to date was an aqueous-ether extract of Salmonella enteritidis of high biological activity. Application of viscometry, osmometry, and light-scattering showed the endotoxin to exist in polydisperse, elongated particles of high molecular weight, with a greater state of aggregation when dissolved in water than when dissolved in dimethylformamide. These results were compatible with those we had obtained by measurements of sedimentation and diffusion. Application of

scanning electron microscopy revealed that the lyophilized preparation had undergone a further stage of aggregation into fibrous structures with marked resemblances to cellulose and certain synthetic fibers.

3. Mr. Jim Cutler, a doctoral candidate in microbiology under Prof. Lorraine Friedman of Tulane University, spent most of the summer of 1971 here studying endotoxin-like substances in the pathogenic fungus, Candida albicans. He demonstrated two distinct toxic components of the fungus, both of which differed significantly from bacterial lipopolysaccharides and that the activity could not be explained by chance contamination with bacterial endotoxin.

4. Dr. K. F. Meyer solicited Dr. Ribi's collaboration in studies on the role of endotoxin in prophylaxis against plague. Dr. Meyer contributed frozen cell pasteof the attenuated Paris "f" strain of Pasteurella pestis grown and harvested by Cutter Laboratories. Several conventional methods of extracting the whole cells gave products containing mainly nucleic acid and nitrogenous contaminants, which were difficult to remove. This problem was circumvented by first preparing cell walls with the aid of the refrigerated pressure cell and extracting the trypsinized and washed cell walls by the hot phenol-water method. With this method we obtained a clean product having the characteristics of a strong endotoxin.

5. As part of a continuing collaboration with Dr. Rudbach in studies of the endotoxin-detoxifying action of serum or plasma, we have provided one of his graduate students, Mr. Kenneth Von Eschen, with facilities, materials, guidance, and assistance. In phylogenetic studies the endotoxin-detoxifying factor was shown to be present in all sera of vertebrates tested, in descending order from primates through Osteichthyes (fishes), except in Chondrichthyes (dogfish shark). It was absent in hemolymph of two representatives of invertebrates (Crustacea). The phylogenetic origin of the factor appeared, therefore, to be in the lower orders of vertebrates. In ontogenetic studies conducted in chick embryos, a slight increase with age in capacity of embryonic serum to detoxify in vitro could be correlated with increased resistance of older embryos to the lethal effect of endotoxin, but the latter increased much more dramatically.

Significance to biomedical research and the program of the Institute:

1. The general relevance of studies of endotoxin tolerance was discussed in last year's report. The current findings indicate that tolerance must be evaluated in terms of specific effects in particular species. The broad mechanisms of induced resistance so far identified--protection by antibody and enhanced phagocytic activity--do not explain the complexity of observed results; consequently, other mechanisms must be involved. The significance of studies of normal endotoxin-detoxifying mechanisms in body fluids can be considered at the same time. It appears self-evident that such mechanisms affect the ability of organisms to deal with a virtually

ubiquitous poison; yet, paradoxically, highly susceptible animals--even young embryos--have these factors in nearly as great supply as much more resistant animals; and invertebrates, which are thought not to be affected by endotoxin, are lacking in the humoral detoxifying factors. These facts require explanation.

2. Investigation of the structure of endotoxin is the special province of this Section and this project. For several years we have sought to identify the essential features of composition and structure of endotoxins, and have devised various means for testing the hypothesis that "endotoxicity" resides more in the physical structure of particles than in any fixed chemical composition. Thus the work of Dr. Cross is valuable for the support it gives our earlier conclusions and the additional information it provides about the behavior of endotoxic molecules in solution. The recent demonstrations of endotoxin-like activity in synthetic double-stranded RNA and in the purified glycopeptide "murein" of streptococci have tended to favor this hypothesis. The substances in Candida sp. responsible for pyrogenic and lethal effects have now been shown to be clearly different from bacterial lipopolysaccharides. Therefore, there is great interest in having the fungal toxins purified and characterized. Any new information on the bases and mechanisms of toxicity would be a fundamental contribution to pathological physiology, with applications to production of biologicals and management of shock.

Proposed course of project:

For the immediate future we propose to continue the associations with Dr. Cross and Dr. K. F. Meyer. It is expected that Visiting Scientist Dr. Ichiro Azuma will introduce a new program of research on bacterial lipids, which will largely occupy the technical assistants; and it is hoped that Dr. Cutler's application for a postdoctoral fellowship to work here on fungal toxins will be funded. Certain projected activities have been curtailed or abandoned for lack of personnel. Technicians who were to have proceeded with studies of the nonendotoxic lipopolysaccharides of Francisella tularensis, and who were to make the preparations for a collaborative study with Prof. C. Weibull (University of Lund, Sweden), have been assigned other duties.

For revitalization of this project, it is imperative that a full-time professional of the regular staff be assigned to it, either to take over Dr. Milner's responsibilities for biological assays or to assume day-to-day supervision of research. The loss of Dr. Rudbach and two technicians has had a seriously crippling effect.

Honors and Awards:

Dr. K. C. Milner

Invited panelist, Airlie House Endotoxin Conference, May 31-June 2, 1972.

Appointment to Editorial Board Infection and Immunity, December 1971.

Appointed to Dissertation Committee for J. E. Cutler, Tulane University (duties fulfilled).

Continuing appointment, Faculty Affiliate (Lecturer in Medical Microbiology), University of Montana.

Invited (with Drs. E. Ribi and R. Parker) to write chapter for Methods in Biochemical Analysis.

Publications:

Rudbach, J. A.: Molecular immunogenicity of bacterial lipopolysaccharide antigens: establishing a quantitative system. J. Immun. 106: 993-1001, 1971.

Milner, K. C., Rudbach, J. A., and Ribi, E.: General characteristics. In Weinbaum, G., Kadis, S., and Ajl, S. J. (Eds.): Microbial Toxins, Bacterial Endotoxins. New York, Academic Press, Inc., 1971, vol. 4, pp. 1-65.

Serial No. NIAID-179
1. Rocky Mountain Laboratory
2. Molecular Biology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The role of morphological elements of microorganisms in immunity and related phenomena

Previous Serial Number: Same

Principal Investigator: E. Ribi

Other Investigators: R. L. Anacker, W. D. Bickel, W. R. Brown, G. Goode, T. J. Meyer, K. C. Milner, R. Parker (NIH Post-doctoral Fellow), and R. F. Smith

Cooperating Units: Drs. B. Zbar and H. Rapp, NCI, NIH, Bethesda, Md.
Dr. W. Brehmer, Robert Koch Inst., Berlin, West Germany
Dr. D. G. Petcoff and Mr. C. J. Filz, Instrument Research Lab., Ivan Sorvall, Inc., Hamilton, Montana
Dr. J. Matsumoto, Research Inst. of Tuberculosis, Osaka, Japan
Mr. S. M. Strain, MIT, Cambridge, Mass.
Dr. S. Kanter, VA Hospital, Palo Alto, California

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	5 1/12			5 1/12
Professional:	3 7/12			3 7/12
Other:	1 6/12			1 6/12

Project Description:

Objectives:

Studies of the fine structure and chemical composition of microorganisms and correlating these attributes with certain biologic properties of cells or cell fractions, and the development and application of new tools and techniques to current problems comprise the activities of this project. This year's studies have been concerned with 1) tumor suppressive

activity of mycobacterial cell wall preparations, and 2) applying microparticulate gel chromatography for isolating and quantitating drugs and their metabolites in body fluids and producing milligram quantities of chromatographically pure biologically active substances, such as bacterial lipids and Δ^9-tetrahydrocannabinol (Δ^9THC) from marihuana and hashish.

Methods employed:

A combination of biophysical, biochemical and immunological techniques has been used. Problems requiring new methodology or specialized uses of the cell fractionator for the preparation of mycobacterial cell walls, or centrifugal microparticulate gel chromatography have been assigned to this project. Tumor immunity induced by mycobacteria and their cellular fractions was studied with a model consisting of transplantable syngenic tumors induced by the water-soluble carcinogen, diethylnitrosamine, in inbred strain 2 guinea pigs.

Major findings:

1. Intradermal inoculation of 10^6 line 10 cells of a syngenic transplantable hepato-carcinoma into strain 2 guinea pigs leads to progressively growing intradermal tumors and metastases to lymph nodes; the animals die within 90 days.

In one assay, mycobacterial cell walls and tumor cells were mixed in vitro and inoculated intradermally. In the second assay, mycobacterial cell walls were inoculated into established intradermal tumors. Results: 1) Tumor growth was suppressed if 10^6 tumor cells were inoculated together with 75 µg BCG cell walls attached to oil droplets. 2) Tumor growth was not suppressed if the tumor cells were given together with BCG cell walls, oil droplets, or oil droplets and BCG cell walls prepared in a way that prevented attachment of cell walls to oil droplets. 3) Injection of 300 µg of cell walls attached to oil droplets into established intradermal tumors caused them to regress and prevented metastases. 4) Injection of oil droplets or Mycobacterium smegmatis cell walls attached to oil droplets into established intradermal tumors did not cause the tumors to regress or prevent development of metastatic tumors.

We have previously shown that BCG cell walls, from which lipids had been exhaustively extracted with organic solvents or treated with sodium hydroxide solution, would not protect mice against aerosol challenge with H37Rv. Fractions of toxic lipid extracts alone or attached to oil droplets also were inactive, but protection was restored if they were recombined with the sodium hydroxide treated "detoxified" cell walls (see findings described under Serial No. NIAID-177). Such preparations were used for tumor immunity studies. Results: 1) Tumor growth was suppressed if 10^6 line 10 tumor cells were inoculated together with 75 µg of "detoxified" BCG cell walls attached to oil droplets. 2) Tumor growth was not suppressed if the tumor cells were combined with toxic lipids alone, or attached to

oil droplets. 3) Tumor suppressing efficacy of "detoxified" cell walls apparently was not enhanced when the toxic lipid fraction was added to them (37.5 µg cell walls plus 37.5 µg lipid). Results of injecting "detoxified" cell walls into established tumors are not yet available.

The development of systemic tumor immunity in guinea pigs in which tumor growth was suppressed after receiving a mixture of oil-treated BCG cell walls and tumor cells was evaluated by intradermal challenge with 10^6 living line 10 tumor cells. The challenge dose was inoculated contralateral to the immunization site. The presence of delayed cutaneous hypersensitivity reactions to tumor cells and prevention of tumor growth indicated that guinea pigs treated with the vaccine had developed systemic tumor immunity.

About 6 X 10^6 living BCG cells must be mixed with line 10 tumor cells to induce tumor regression. This large dose produced a large, ulcerating lesion at the site of inoculation; the lesion healed after about one month and left a scar. Although the effective dose of 75 µg of either untreated or NaOH-treated cell walls produced a minimal local lesion, kinetic studies of the histopathologic changes produced by cell wall vaccines are in progress.

2. Continued development and application of centrifugally accelerated chromatography on gels prepared with ultrafine silica (Centri-Chrom) have been emphasized. This year the technique was used for the detection and quantitation of important components in marihuana and hashish and their metabolites in urine after ingestion of the drug. We obtained from Dr. Saul Kanter extracts of urine from a volunteer who had ingested 2 doses of 20 mg of hashish 12 hours apart. Urine was collected during a 12-hour period after the second dose. In 63 ml of urine extracted with petroleum ether at pH 5.5 we detected not only the cannabinoid components formed in the host (metabolites and/or conjugates) but also the native hashish components, some of which were present in low concentration.

In order to perform valid experiments on the effects of the psychomimetically active component, Δ^9THC, and the formation of metabolites, as well as to compare natural and synthetic Δ^9THC, it is necessary to start with a highly purified product. None of the several lots of synthetic standards received by us has been free of contaminants. In one standard, we detected three major and at least two minor contaminants, and the pattern of another showed the presence of at least nine components. A gas chromatogram of the latter indicated the presence of only five components.

Because of the need for working quantities of chromatographically pure components, we enlarged the diameter of the chromatographic tubing to 18 mm, but retained the same 65-mm length in an attempt to increase the yield. Columns were packed with gel by applying centrifugal force and, to facilitate monitoring of the eluate, development was accelerated by pressure (80 to 100 psi) instead of by centrifugation. Monitoring was accomplished

with the aid of Chromatronix microflow cell at 280 nm in an LKB Uvicord coupled to a Heath recorder, whose sensitivity had been increased by shunting the signal input terminals with a resistor and capacitor in parallel. Fractions collected at the absorption peaks were found to contain single bands when rechromatographed with the 3-mm microanalytical Centri-Chrom apparatus. By simultaneous use of two 18-mm diameter columns, about 1 mg of purified Δ^9THC can be gleaned per run from either synthetically prepared Δ^9THC or marihuana plant distillates.

Whereas the method for identification of marihuana and hashish constituents in "street" samples with Centri-Chrom, as published in Science, was shown to be an improved qualitative analytical tool, a reliable quantitative method for research and forensic application was still needed. Such a quantitative technique has now been developed. We apply 10 to 50 µg of marihuana or hashish petroleum ether extract on top of an 8-mm gel column and monitor the eluate through a UV-microflow cell coupled with a Heath recorder as described above. The concentration of the three major constituents, Δ^9THC, cannabinol, and cannabidiol, in which forensic workers are primarily interested was determined by collecting the eluate from the beginning to the end of each UV peak, staining it by adding Echtblausalz B, and then subjecting it to colorimetric analysis. It is estimated that the presence of 10 µg of Δ^9THC can be determined with an accuracy of $\pm$ 5% by the colorimetric method.

Pressure elution chromatography was also applied to the separation and identification of 4-4'-diamino-diphenyl-sulfone (DDS), a drug used in the chemotherapy of leprosy, and its metabolites, N-acetyl-DDS (NADDS), and N,N'-diacetyl-DDS (DADDS). Mixtures of these three compounds had been resolved previously with the aid of the 3-mm Centri-Chrom apparatus. Each component formed a band which contained 10 to 20 µg, a concentration needed to make them visible by charring. When eluted by pressure from 8-mm columns and the eluate monitored with a UV flow cell, microgram amounts could be separated and detected. The area under the peak would indicate the amount of a component present.

Significance to biomedical research and the program of the Institute:

Zbar, Rapp and Bartlett recently reported that intradermal growth of a transplantable syngenic line 10 hepato-carcinoma in normal guinea pigs is suppressed if the tumor cells are inoculated together with living BCG. Attempts to produce tumor immunity by injection of BCG at one site and tumor cells at another failed. In immunotherapy experiments in which viable BCG was injected into established skin tumors, more than half of the animals were cured of both the skin tumor and the metastases. Results stressed the need for a direct contact between BCG and tumor antigen for effective immunotherapy. The line 10 tumor offers a challenge because of its "low" tumor specific antigenicity or poor immunogenicity. Unlike that

seen with line 1 tumors, intradermal growth and regression do not lead to development of systemic tumor immunity and, hence, the participation of a powerful adjuvant is required to produce effective systemic immunity.

Attempts to immunize guinea pigs against line 10 tumor cells by injecting a mixture of tumor cells and nonviable BCG (heat-killed) or BCG extracts have failed. When viable BCG was substituted for complete Freund's adjuvant in the mixture, it was effective in suppressing growth of the viable tumor cells injected, but was not effective in inhibiting growth and metastasis of simultaneous 10^6 tumor cell challenge. Several investigators have stressed in the past that for treatment of cancer patients, however, nonliving mycobacterial preparations with tumor-suppressive properties would be preferable to living BCG. As long as a decade ago, it has been stated by Drs. Paul Lemonde and Margarida Clode-Hyde that "It might be possible to devise some form of treatment that would eliminate tuberculous disease (the infectious process of BCG) while retaining the beneficial influence of BCG". We now report that small quantities of "intact" cell walls from BCG, as well as cell walls from which the major toxic components had been removed, when attached to minute oil droplets, not only possess potent tumor suppressive activity but also can be used to cure both established skin tumors and metastatic tumors.

The minimal effective dose of detoxified cell walls has not been established but 37.5 µg depressed tumor growth and induced systemic tumor immunity; in fact, when these animals were challenged with 10^6 living tumor cells, the resulting delayed cutaneous reaction—thought to be a criterion for established immunity—was far more extensive than that seen in animals immunized with 150 µg of "intact" cell wall vaccine or with 6×10^6 viable units of BCG. Superficially at least, and contrary to our expectations, it would appear that the activity necessary to enhance tumor immunity resides in the "detoxified" cell wall.

Our knowledge of antituberculous immunity is still very incomplete and, according to Dr. Mackaness, it would be ironic if the use of BCG or components isolated from it as immunotherapeutic agents against cancer were to teach us more about the immunological properties of BCG than we have learned in half a century of its use as a prophylactic agent for the prevention of tuberculosis.

Modifications of the Centri-Chrom technique, wherein movement is accelerated by pressure in large columns and the separation monitored quantitatively, has for the first time enabled the production of working quantities of pure cannabinoids. The methods should have wide application in forensic medicine and in pharmacologic studies of these drugs, as well as in research on complex lipids of microorganisms (see NIAID-177).

Proposed course of project:

1. The finding that the adjuvant of the tubercle bacillus responsible for enhancement of tumor immunity resides in the detoxified cell wall creates a new field of investigation much too extensive for a single group of investigators. Our choice is restricted by the resources at RML and the extent to which collaboration is possible with NCI. An immediate attempt will be made to establish the minimum structural attributes essential for adjuvant action. We also plan to determine whether cell walls from other species and strains of Mycobacteria possess this activity; cell walls from Nocardia and Corynebacteria also will be examined. Attempts will be made to prepare tumor cell membranes and/or to isolate tumor specific antigen(s) which then will be combined directly with the adjuvant-active BCG cell wall in tests for immunogenicity. Such a vaccine would not possess a tumorigenic hazard associated with the use of living tumor cells.

Dr. John Coe will participate in attempts to determine the role of immunoglobulins involved in the tumor immune reaction elicited by the action of the BCG adjuvant.

Experiments with two tumor lines in C3H mice, one strongly antigenic and one poorly antigenic, will be carried out to determine the inter-relationships between adjuvant action and antigenicity of the treated tumor.

2. Isolation, purification and structural identification of metabolites of Δ^9THC from body fluids with the aid of accelerated microparticulate gel chromatography combined with infrared spectroscopy, mass spectrometry and nuclear magnetic resonance.

Analysis of drugs by centrifugal microparticulate bed chromatography will be extended to include the identification and characterization of opium alkaloids.

Publications:

Petcoff, D. G., Strain, S. M., Brown, W. R., and Ribi, E.: Marihuana: identification of cannabinoids by centrifugal chromatography. Science 173: 824-826, 1971.

Anacker, R. L., Simmons, J. H., and Ribi, E.: Separation of sugars by centrifugal microparticulate bed chromatography. J. Chromatogr. 62: 93-97, 1971.

In press:

Zbar, B., Rapp, H. J., and Ribi, E. E.: Tumor suppression by cell walls of Mycobacterium bovis (BCG) attached to oil droplets. J. Nat. Cancer Inst.

Serial No. NIAID-180
1. Rocky Mountain Laboratory
2. Serological Technology Sect.
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The encephalitides

Previous Serial Number: Same

Principal Investigator: L. A. Thomas

Other Investigators: None

Cooperating Units: Dr. Michael Goloff, (EIS) Montana State Department of Health and Environmental Sciences, Helena
Dr. David B. Lackman, Microbiology Laboratory, Montana State Department of Health, Helena

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	11/12			11/12
Professional:	2/12			2/12
Other:	9/12			9/12

Project Description:

Objectives:

Major objectives of this project are: 1) to elucidate the role of garter snakes in the overwintering of WEE virus, 2) to determine arbovirus activity in the area as measured by presence of antibodies in human and/or animal sera, and 3) to characterize the antigenic relationships of viruses isolated from field material, especially ticks.

Methods employed:

Standard procedures are used in all phases of the study, including preparation of antigens and immune sera, isolation and identification of viruses and serological tests (hemagglutination, hemagglutination-inhibition (HAI), complement fixation (CF), and neutralization).

Major findings:

1. Viremia and antibody studies of garter snakes experimentally infected with WEE virus. Twenty garter snakes from the Bear River Refuge, Utah, and 8 from Hamilton, Montana, without detectable WEE antibodies, were inoculated intraperitoneally with 1 million suckling-mouse LD_{50} of WEE virus on May 28, 1971, and held at room temperature for study. Viremia of varying duration was demonstrated in 15 (75%) of the snakes from Utah and in 7 (88%) of those collected locally. A second inoculation of WEE virus (same dose and route) was given 3 months later to this group of snakes and to previously uninoculated Utah and local snakes. We were unable to demonstrate viremia in 18 previously inoculated Utah snakes, or in the 1 local snake, although it was detected in 2 of 6 previously uninoculated snakes from Utah and 4 of 4 of those from Hamilton. All surviving snakes from the study (20 from Utah, and 1 from Hamilton) were bled out October 28, 1971, and tested for HAI and neutralizing antibodies. HAI titers of 1:10 were demonstrated in 4 snakes and 1:20 in 1 snake—these titers were too low to be considered significant. Neutralizing antibody titers likewise were low and were regarded as insignificant.

2. WEE and SLE virus activity in Montana. In collaboration with the Montana State Department of Health, we provide limited laboratory support in their surveillance of these diseases. Activity of both viruses was at a low level the past year. Neither virus was recovered from a limited number of mosquitoes tested (9 Culex tarsalis and 150 other species) during early August. However, tests of serums from sentinel chickens bled periodically indicated WEE virus activity between August and early October. WEE was confirmed in only 1 of 27 suspected cases in man; 1 of 2 horses thought to have WEE had an HAI titer of 1:80 in both paired serums. WEE HAI titers greater than 1:10 were detected in only 7% of 326 serums collected at a heart clinic in Glasgow, Montana. These findings indicate a low level of WEE activity in the area for quite some time.

3. EEE HAI antibodies in experimentally infected dogs. Dr. J. F. Bell (RML) inoculated 30 dogs with EEE virus and submitted pre- and multiple post-inoculation serums and cerebrospinal fluid for HAI antibody determinations. HAI antibodies were detected in postinoculation serums of all dogs but not in the spinal fluid collected at corresponding bleeding dates.

Significance to biomedical research and the program of the Institute:

The role of garter snakes in the ecology of WEE virus continues to be questionable in light of findings the past year. Standard methods for demonstrating WEE infection in garter snakes are not adequate. All studies or reports of antibody response in cold-blooded vertebrates should be carefully evaluated for methods and techniques used.

Proposed course of project:

Study of WEE virus in garter snakes will not be pursued during the coming year unless a "different" method of determining antibody is employed.

A collaborative study is proposed to investigate the role of leeches in the transmission of certain arboviruses, Japanese B encephalitis virus in particular.

We will continue to assist the Montana State Department of Health in the isolation and identification of WEE and SLE viruses from mosquitoes and by antibody determinations in serums.

Publications:

Newhouse, V. F., Burgdorfer, W., and Corwin, D.: Field and laboratory studies on the hosts and vectors of the snowshoe hare strain of California virus. Mosquito News 31: 401-408, 1971.

Serial No. NIAID-181
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Tick-borne disease agents

Previous Serial Number: Same

Principal Investigators: C. M. Clifford, C. E. Yunker, and L. A. Thomas

Other Investigators: J. Cory, L. E. Hughes, and J. E. Keirans

Cooperating Units: Dr. J. Casals, Yale Arbovirus Research Unit, New Haven
Mr. E. Easton and Dr. R. Goulding, Oregon State University, Corvallis
Dr. J. E. George, Texas Technological College, Lubbock
Mr. R. Gresbrink, Oregon State Board of Health, Portland
Mrs. Margaret Sweeney, Idaho State Department of Health, Boise

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	6 3/12			6 3/12
Professional:	2 3/12			2 3/12
Other:	4			4

Project Description:

Objectives:

To determine the incidence, distribution and ecology of tick-borne diseases that influence man and domestic animals in the United States. Of equal importance is the investigation of the vector relationships of the increasing number of viruses known or thought to be associated with ticks.

Methods employed:

Field and laboratory approaches include conventional and specialized entomological, ecological, virological and immunological techniques.

Major findings:

1. Viruses and ixodid ticks. Thirty-six strains of viruses isolated from Ixodes uriae collected from coastal Oregon during 1970 were identified as to antigenic group. (I. uriae, a seabird-parasite, was previously shown to harbor Tuleniy [Three Arch] virus, a group B agent closely related to Japanese B encephalitis.) The 1970 isolates were shown to be related to viruses in 4 antigenic groups: "B", Kemerovo, Uukuniemi, and Sakhalin. Identity of the isolates was demonstrated in at least 2 of 3 serological tests performed. The 5 strains of the B group were identified as Tuleniy virus and the 12 related to Sakhalin were shown to be this virus. The 7 strains of the Kemerovo-like virus were not identical to any of the known agents of this group and are believed to represent a virus new to science. Specific identity of the 13 Uukuniemi-like viruses has not yet been established. Presence of more than one virus in a single pool was seen in 5 pools and 7 additional strains were unidentifiable by reason of low titer. In addition, a pool of 7 I. uriae collected on St. Lawrence Island in 1971 yielded the new virus of the Kemerovo group mentioned before. This is the first report of a Kemerovo-group virus from ixodid ticks in the United States, and the first reported instance of Sakhalin virus or a Uukuniemi-like agent in the U.S.

The new virus in the Kemerovo group was characterized. This virus, called Yaquina Head for a locality in Oregon from which it originated, is deactivated by diethyl ether, capable of passing through filters of 220 mμ average pore diameter (apd) but not through those of 100 mμ apd, and infective for newborn but not weanling mice. In Vero cells it produces a cytopathogenic effect and forms plaques. Haemagglutinating antigen could not be demonstrated in sucrose-acetone extracts of infected mouse brain at various pH's and temperatures. In complement-fixation (CF) tests, virus antigen did not react with antisera of 91 viruses but did react with that of Kemerovo virus. In the reciprocal test Yaquina Head antiserum reacted slightly with Kemerovo and Chenuda viruses. A similar relationship was seen in suckling mouse and tissue culture cross-neutralization tests. In the tissue culture neutralization test a slight degree of reciprocal relationship between Yaquina Head and Tribeč viruses was seen. A manuscript is in preparation describing these findings.

I. uriae were also collected from two areas on the Oregon coast during the summer of 1971. These collections consisted of 31 ♂♂, 40 ♀♀, 15 NN from Right Rock Yaquina Head, Lincoln Co., and 134 ♂♂, 297 ♀♀, 2 NN, 75 LL from Goat Island, Curry Co. Four strains of virus were isolated from

12 pools of the Yaquina ticks (1 in tissue culture and 3 in suckling mice) and 12 were recovered from 34 pools of the Goat Island material (3 in tissue culture and suckling mice, 1 in tissue culture only and 8 in suckling mice). These strains are being identified.

Studies have been initiated to determine if Culex tritaenorhynchus, the commonest vector of Japanese B encephalitis virus in Japan, can be infected with the related Tuleniy virus from North America and transmit it to susceptible hosts. C. tritaenorhynchus was obtained from Walter Reed Army Medical Center and successfully colonized. Viremias of 3.5 to 3.7 logs were demonstrated in wet chicks 24 to 48 hr after intraperitoneal inoculation of the virus. An adequate threshold of infection was demonstrated in mosquitoes that had fed on viremic chicks. Further experiments in progress are designed to determine the infection rate of mosquitoes and if transmission to susceptible hosts will occur.

Viruses and argasid ticks. In 1971 a large colony of Ornithodoros sp. nr. capensis was discovered on a nearly inaccessible, sheer cliff serving as a nesting site for the common murre, Uria aalge. This colony was located on the mainland at Cape Lookout, Oregon. Twenty-three virus isolations were made from 61 pools of these ticks. Results of preliminary tests indicate that more than one virus is involved, and at least some strains belong to the Hughes group, closely related to Punta Salinas virus (from O. amblus in Peru).

2. Studies with Colorado tick fever (CTF) virus. In a continuing program with the Oregon State Department of Health of surveillance of campsites and picnic areas, 166 and 68 pools of Oregon ticks, mostly Dermacentor andersoni, were tested in suckling mice and African green monkey-kidney cell line (Vero) cells, respectively. Eleven isolates of CTF were recovered and these were confirmed by mouse and/or plaque-reduction neutralization tests.

Last year studies were initiated to determine the duration of the association of CTF virus with circulating blood cells of rats and mice. We reported that virus was detectable in red blood cells of both rats and mice for 45 days. This experiment has been concluded and it has been demonstrated that CTF virus can be detected in association with red blood cells of rats and mice for 50 and 60 days, respectively. A manuscript is being prepared on this subject and should be published during this year.

In collaboration with the Idaho State Department of Health, we tested acute blood samples from febrile patients having recent history of tick-bite. Samples collected by physicians and mailed to this laboratory were inoculated into suckling mice and Vero cells. Viruses so isolated were identified by plaque-reduction neutralization test. Fifteen of 59 bloods yielded CTF virus. Of these, 13 isolations were made in Vero cells

and 2 in mice. Most tissue culture isolates were detected by plaque formation rather than by cytopathogenic effect (CPE). One isolate proved to be influenza virus, as shown by CF reactions. In a previous report we demonstrated that Vero cells are superior at least to the RML strain of Swiss white mice for the detection of CTF virus from rodent bloods and naturally infected ticks. The present findings indicate the same to be true for human bloods.

3. Assay systems for tick-borne viruses. Newly isolated tick-borne viruses plus addition to our collection of some strains we previously lacked have enabled us to document tissue culture reactions of 19 viruses since last report. This brings the total to 68 viruses tested in one or more mammalian cell culture systems. Plaques were produced in Vero cells by the following tick-borne viruses: Omsk hemorrhagic fever virus, Hypr strain of tick-borne encephalitis, Sapphire I, Lipovnik, Tribeč, Yaquina Head, Sixgun City, Grand Arbaud, Qalyub, Thogoto, and Green Kure; also by the following (not tick-borne), Israel turkey meningoencephalitis, Baku, Modoc, Western equine encephalitis, Trivitattus, dengue 2, St. Louis encephalitis, Itaqui, Tacaribe, and Myxoma. CPE was produced in Vero cells by Ganjam, Yaquina Head, and Quaranfil viruses. Both BHK-21 and PK-H13 cells were tested as alternative detection and quantitation systems for certain virus. In the former cell-line, positive results were obtained with Kemerovo (plaques and CPE), Sapphire I and Sixgun City (plaques), Nyamanini, Langat, and Green Kure (CPE); in the latter, with Kemerovo and Powassan (plaques) and Dugbe and Tribeč (CPE). Approximately 77 percent of 41 tick-borne viruses are thus detectable by their effect on mammalian tissue culture systems. Of particular importance to this program is that we are able to plaque the North American virus, Powassan. Another related tick-borne virus from this continent, Tuleniy, of potential epidemiological significance, has thus far resisted our efforts to plaque it.

Significance to bio-medical research and the program of the Institute:

The genus Ixodes plays an important role in the transmission of viruses that cause illness in man, e.g. RSSE, Central European TBE, Louping Ill, Kemerovo, Omsk haemorrhagic fever and Powassan viruses. They are also important vectors of pathogenic viruses to domestic livestock. Our demonstration of a multiplicity of viruses in Ixodes ticks in North America is of potential epidemiological significance.

Our most recent findings continue to emphasize the tremendous geographic distribution of viruses associated with ixodid ticks, i.e. the occurrence of Kemerovo group, Sakhalin and Uukuniemi-like viruses in western North America.

Japanese B encephalitis (JBE), a severe, sometimes fatal disease of humans in the Orient, has not been reported from the United States.

However, it is known that many species of North American mosquitoes are capable of transmitting JBE virus. In addition, the close relationship of this virus to Tuleniy virus from Oregon seabird ticks has invited speculation as to the evolution of the latter from a mosquito-borne JBE-ancestral strain and has posed the question of true vector relationships of Tuleniy virus. For these reasons it is essential to know if American strains of Tuleniy virus can be transmitted by an efficient vector of JBE virus such as C. tritaenorhynchus.

Because of the low titers and unstability of many tick-borne viruses, isolation and assay systems more sensitive and reliable than conventional laboratory animals must be devised. Hence our continued emphasis on tissue culture studies. For example, we have shown that CTF virus from human bloods and certain Kemerovo-group agents are more easily isolated in Vero cells than in suckling mice.

Although in previous years we have tested sizeable numbers of Ornithodoros sp. near capensis from offshore Oregon bird islands, viruses have not been isolated. The present finding of at least one arbovirus in these ticks on mainland Oregon is therefore unique. Should this virus be confirmed as Punta Salinas, a suspected human pathogen, this will offer another striking instance of intercontinental distribution of viruses associated with seabirds.

Proposed course of project:

Studies on the distribution and incidence of tick-borne viruses in the Northwest will continue, again, mainly through collaborative efforts with the Oregon and Idaho State Health Departments, and Oregon State University. This year emphasis will be placed on attempts to isolate more strains of Rocky Mountain spotted fever (RMSF) rickettsia from the Tillamook area on the Oregon coast. The strains from this area are of interest because they have been derived from I. pacificus and occur outside the range of the normal vectors of RMSF.

Isolation and characterization of viruses associated with ticks will continue. Emphasis will be placed on the antigenic comparison of viruses already on hand. With the future of the major North American arbovirus reference center in doubt it is possible that our progress with tick-borne viruses could be severely hampered. Therefore, we will continue to obtain stocks of tick-borne and related viruses and to prepare serological reagent for these.

Efforts will be continued to develop tissue culture techniques to facilitate identification of tick virus strains as they are recovered.

Publications:

Clifford, C. M., Yunker, C. E., Thomas, L. A., Easton, E. R., and Corwin, D.: Isolation of a group B arbovirus from Ixodes uriae collected on Three Arch Rocks National Wildlife Refuge, Oregon. Amer. J. Trop. Med. 20: 461-468, 1971.

In press:

Yunker, C. E., Clifford, C. M., Thomas, L. J., Cory, J., and George, J. E.: Isolation of viruses from swallow ticks, Argas cooleyi in the southwestern United States. Acta virol.

Serial No. NIAID-183
1. Rocky Mountain Laboratory
2. Comparative Pathology and Arbo and Chronic Viral Diseases Sections
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Viruses as a probable cause of certain chronic diseases of man

Previous Serial Number: Same

Principal Investigator: W. J. Hadlow

Other Investigators: R. K. Bergman, J. E. Coe, R. C. Kennedy, G. C. Lavelle, J. J. Munoz, and R. E. Race

Cooperating Units: USDA, Animal and Plant Inspection Service, Veterinary Services: Dr. A. L. Klingsporn, Hyattsville, Md., and Mission, Texas
Dr. K. K. Takemoto, LVD, NIAID, NIH, Bethesda, Md.
Dr. C. M. Eklund, Minneapolis, Minnesota

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	17 6/12			17 6/12
Professional:	4 7/12			4 7/12
Other:	12 11/12			12 11/12

Project Description:

Objectives:

From a study of naturally occurring slow viral diseases of domestic animals, 1) to obtain an understanding of the mechanisms by which viruses cause protracted diseases, and 2) to develop working concepts and investigational procedures that will facilitate determining whether viruses are likewise causally related to some chronic diseases of man.

Methods employed:

Four diseases that represent the models for continued study include: 1) scrapie, a subacute encephalopathy of sheep, 2) a comparable neurologic disease of ranch mink, 3) Aleutian disease, a protracted systemic disease of mink, and 4) progressive pneumonia, a chronic proliferative pulmonary disease of sheep. Clinicopathologic, virologic, and immunologic features are studied in an effort to gain an understanding of the natural history and pathogenesis of each disease. Characterization of the causative viruses is of secondary concern, and is pursued within the limits imposed by the availability of personnel competent to do this.

Major findings:

1. Scrapie

a. The disease process. Obtaining a better understanding of the natural history and pathogenesis of this disease in sheep was emphasized. Material obtained from the USDA Scrapie Field Trial, Mission, Texas, forms the basis for this study.

Data obtained so far on the quantitative distribution of virus in sheep (mainly of the Suffolk breed) affected with the natural disease now provide a clearer picture of the virologic events in pathogenesis. As reported previously, the greatest concentrations ($10^{-4.5}$ to $10^{-6.5}$) of virus were found in the target organ—the central nervous system, especially the cerebellar cortex, brain stem, and subcortical gray masses. Results of viral assays during the last 2 years indicate that titers in lymphocytic tissues (spleen, tonsil, and both peripheral and visceral lymph nodes) are 10^{-2} to 10^{-3}. Titers in the ileum and upper colon, both of which contain lymphocytic tissue, are about the same. Much less virus (10^{-1}) was detected in the lower colon. Virus was barely detectable in the cerebrospinal fluid. In some sheep, titers of at least 10^{-1} were found in the nasal mucosa. These results, though still incomplete, are consistent with the view that under natural conditions virus most likely enters the body via the nasopharynx or the lower gastrointestinal tract. It then spreads to regional lymph nodes, replicates here, and then spreads to other lymph nodes and the spleen. After many months, virus appears in the central nervous system. Here it slowly increases in amount until clinical signs of disease appear.

To provide additional data supporting this view, 6 newborn lambs from high-risk Suffolk families (85% of the progeny become affected with scrapie by 4 years of age) were examined for virus in various tissues. Virus was not detected (by mouse inoculation) in any of them or in colostrum of the dams. Thus, although the familial occurrence of the disease is often interpreted as evidence of its vertical transmission (from dam to fetus in utero), our efforts to substantiate this by isolating virus from fetuses (reported last year) and newborn lambs so far have failed to do so.

In view of this, and the epidemiologic observations at the Scrapie Field Trial, infection most likely occurs early in the postnatal period, perhaps during the first 4 months after birth. Accordingly, efforts are being made to detect virus in other high-risk Suffolk lambs varying in age from a few month to 2 years, i.e., at intervals during the long incubation period. To date, tissues from 2 yearling and 3 two-year-old lambs, all clinically normal when killed, have been inoculated into mice. So far (13 months later), virus has been detected in the ileum and suprapharyngeal lymph node of 1 yearling and in the spleen of 1 two-year-old. These preliminary results are encouraging. They indicate that this general investigational approach is providing virologic data that will allow us to assemble a more complete picture of the pathogenetic events and the mode of transmission in the natural disease.

b. The virus. As mentioned last year, an additional effort was made to determine the susceptibility of mink to scrapie virus. Although it may be too early to assess the results of this experiment, brain suspensions of known high virus titer from 1 Cheviot and 2 Suffolk sheep affected with natural scrapie have nevertheless failed after 17 months to cause disease in pastel mink inoculated intracerebrally. This is in contrast to the findings of others who reported the occurrence of a polio-encephalopathy in mink 12-14 months after intracerebral inoculation with a brain suspension from a naturally affected Suffolk sheep.

2. Mink encephalopathy. As reported last year, a disease essentially indistinguishable from scrapie occurred in goats inoculated intracerebrally with mink encephalopathy virus (Idaho isolate). To date, 10 of 19 goats have become affected 29-40 months after inoculation. By contrast, only 1 of 19 Cheviot sheep similarly inoculated has become affected so far, 48 months after inoculation. Brain suspensions from one affected goat caused typical encephalopathy in pastel mink 7 months after inoculation. Similarly inoculated RML Swiss mice are still normal after 8 months. These results still leave unanswered the relationship of mink encephalopathy virus and scrapie virus.

3. Aleutian disease (AD)

a. The disease process. In the continuing study on the temporal distribution of virus in experimentally infected sapphire mink, the main new observation was the consistently high titers (10^{-4} to 10^{-7}) of virus detected in bone marrow. These concentrations of virus roughly paralleled those found concurrently in the intestine. From this and other observations made to date, it is obvious that in extremely susceptible mink, e.g., sapphires, many tissues support continued replication of virus in high titer, beginning early in the course of the infection. In most tissues, such replication is unaccompanied by any significant pathologic change.

Presumably, the continued replication of virus in any one of several tissues, e.g., lymph nodes, spleen, and bone marrow, is responsible for the viremia that characteristically persists in extremely susceptible mink.

b. Studies on immunoglobulins and immune responses of normal and AD affected mink. See NIAID Project 175.

c. The virus. Mainly because methods for doing so are cumbersome, comparative studies on different isolates of AD virus have not been done here or elsewhere. To date our studies have been confined to the Pullman isolate. To obtain some comparative data, a preliminary study was made on the behavior of the Utah isolate (obtained from David Porter, U.C.L.A.) in RML mink. Differences were observed, particularly in the response of the pastels. For example, all pastels (6 in each group) inoculated intraperitoneally with a 220 mu or a 100 mu Millipore filtrate of an infected liver suspension became affected with AD. In most, the disease followed the usual protracted clinical course observed in this color phase. Unexpectedly, all mink (3 sapphires and 6 pastels) inoculated with a 50 mu Millipore filtrate of the same liver suspension remained free of any evidence of AD. These results are in sharp contrast to those obtained at RML with the Pullman isolate. For example, with this isolate only 30-50 percent of the pastels become affected with AD, even when they are inoculated with large amounts of virus. Moreover, this isolate readily passes a 50 mu Millipore membrane. The occurrence of transient signs of acute disease 2 to 3 weeks after inoculation in mink (sapphires and pastels) that received the 220 mu filtrate was another difference. Reasons for this were not apparent, but the observation suggests that some other pathogen may have been present in the liver suspension and was removed by the 100 mu membrane.

Ostensibly, these preliminary results indicate that the Utah isolate is more virulent than the Pullman isolate for pastel mink. Comparative titrations of the 2 isolates, now in progress, should provide a better understanding of the differences observed. Failure of the Utah isolate to pass a 50 mu membrane is most disturbing. Further filtrations will be done to resolve this glaring discrepancy.

4. Progressive pneumonia

a. The disease process. As mentioned last year, variation in the susceptibility of different breeds of sheep to the experimentally induced disease has hampered studies on its pathogenesis. Observations this year suggest that the dairy goat (mainly of Saanen breeding) may be more uniformly susceptible. For example, at least half of the goats inoculated intravenously with a lung suspension of virus were affected at the end of 4 years. Far fewer sheep became affected during the same period.

In contrast to our findings in sheep, pulmonary changes in affected goats may be far advanced and still not evident clinically. Although other differences probably exist, these results are nevertheless sufficiently encouraging to allow at least a pilot study on the pathogenesis of this disease.

b. Antibody responses. Serums from 21 sheep collected at varying intervals after they were inoculated with a suspension of progressive pneumonia virus were examined by Dr. Takemoto with his indirect fluorescent antibody technique. Antibody was detected in only 4 sheep, all at 1:5 dilution. These discouraging results, and our failure to detect complement fixing antibody with the antigen used here indicate that much work still needs to be done to find a workable serologic tool to augment studies on pathogenesis.

Significance to biomedical research and the program of the Institute:

In recent years, increasing attention has been focused on the significance of persistent viral infections in the genesis of disease in man and animals. As one distinctive group of such infections, the slow viral diseases of animals have been of prime importance in delineating some of the pathogenetic mechanisms associated with viruses that persist in readily detectable form. Moreover, their study has provided the basis for the belief that certain protracted diseases of man may likewise result from persistent viral infection. This is particularly well exemplified by scrapie and mink encephalopathy. Observations on them pointed the way to our present understanding of the fundamental nature of such heretofore etiologically-obscure human diseases as kuru and Creutzfeldt-Jacob disease. In fact, these 4 neurologic diseases share so many features that clarification of their etiologic interrelationship is now of great concern. Although AD does not have a known counterpart in man, the pathogenetic mechanisms involved in its evolution should help explain the genesis of some renal glomerular diseases affecting man. Similarly, the pathogenetic phenomena observed in progressive pneumonia should have analogies in lymphoreticular proliferative disorders of man. For these reasons, the study of these slow viral diseases of animals represents an exciting new frontier for both scientists and institutes concerned with infectious disease.

Proposed course of project:

Although certain adjustments will have to be made to accommodate changes in personnel, the main thrust of the project will remain the same during the coming year. Laboratory studies on the causative viruses will be greatly curtailed because of Dr. Lavelle's transfer to Bethesda. Studies on pathogenesis will be expanded now that Dr. T. A. Jackson has joined the Comparative Pathology Section. Also, because of other additions to the RML staff, immunologic aspects of the slow viral diseases will receive more attention.

In general, the following studies will be of main concern:

1. More sheep from the Scrapie Field Trial will be examined for virus to provide a better understanding of the pathogenesis of the natural scrapie.

2. Virologic studies on mink encephalopathy will be continued.

3. In cooperation with the Immunology Section, studies on the pathogenesis of Aleutian disease will be continued with greater emphasis on the immunologic events.

4. A pilot study on the pathogenesis of progressive pneumonia in goats will be initiated.

Publications:

Lopez, C., Eklund, C. M., and Hadlow, W. J.: Tissue culture studies of the virus of progressive pneumonia, a slow infectious disease of sheep. Proc. Soc. Exp. Biol. Med. 138: 1035-1040, 1971.

Lavelle, G. C., Sturman, L., and Hadlow, W. J.: Isolation from mouse spleen of cell populations with high specific infectivity for scrapie virus. Infect. Immun. 5: 319-323, 1972.

Bergman, R. K., Lodmell, D. L., and Hadlow, W. J.: A technic for multiple bleedings or intravenous inoculations of mink at prescribed intervals. Lab. Anim. Sci. 22: 93-95, 1972.

In press:

Eklund, C. M. and Hadlow, W. J.: Slow viral diseases. In Critical Review Journal, Chemical Rubber Co. In press.

Serial No. NIAID-184
1. Rocky Mountain Laboratory
2. Allergy and Immunology
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Biological properties of Bordetella pertussis antigens

Previous Serial Number: Same

Principal Investigators: J. J. Munoz and R. K. Bergman

Other Investigators: J. F. Bell and W. J. Hadlow

Cooperating Units: Dr. Alan Blaskett, Commonwealth Serum Laboratories, Parkville, Victoria, Australia
Mr. C. J. Filz, Ivan Sorvall, Inc., Hamilton, Montana

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 4/12		4/12	3 8/12
Professional:	11/12		4/12	1 3/12
Other:	2 5/12			2 5/12

Project Description:

Objectives:

1) To purify and identify biologically active substances from Bordetella pertussis, and 2) to study the mechanism of action of the histamine sensitizing factor (HSF) and the protective antigen (PA) and to elucidate the adjuvant action of B. pertussis.

Methods employed:

Standard immunological, pharmacological, biochemical and bacteriological techniques and physicochemical methods of fractionating biological materials are used.

Major findings:

1. Reaginic type of antibody in mice (Munoz and Bergman). Bordetella pertussis extracts made in this laboratory have been shown to stimulate the production in mice of γE-like antibody to protein antigens such as hen's egg albumin (Ea). This antibody was partly purified by DEAE cellulose column chromatography and separated from the $7S\gamma_1$ responsible for induction of the so-called 2-hr PCA. The γE-like antibody with specificity to B. pertussis antigens is also produced in mice receiving 50 gamma of our alkaline saline extracts (SE) from B. pertussis. Although specific antisera to γE-like antibody have been produced in guinea pigs as judged by the neutralization of the ability to induce 72-hr PCA, we have not been successful in demonstrating precipitin reactions or binding of antigen by radioimmunodiffusion tests with these antisera. This has led us to believe that the concentration of γE-like immunoglobulin is extremely low, even in our semipurified and concentrated preparations. Further efforts will be made to develop a precipitin test for this new mouse immunoglobulin.

2. Further attempts to purify HSF (Munoz). We have continued our efforts to purify HSF from B. pertussis. Some problems that have been encountered are low recovery of HSF and the insolubility of some of the semipurified preparations. HSF, extracted from acetone-treated cells by means of 0.05 M sodium pyrophosphate in 1 M NaCl at pH 8.5, fractionated in hydroxylapatite columns, with promising results. By subjecting active fractions from these columns to electrophoresis in starch blocks, we have obtained soluble HSF, free from most of the antigens demonstrable by immunodiffusion test. The recovery is still rather poor and we are presently attempting to improve the yield of HSF. An increased yield of HSF was obtained by extracting B. pertussis cells with 1 M NaCl and 4 M urea. Further work with this method will be done.

3. Relationship of HSF to protective antigen, lymphocytosis promoting factor, and adjuvant activity of B. pertussis extracts (Munoz, Bergman, and Blaskett). A study has been initiated to find whether HSF is involved in protecting mice against i.c. challenge with virulent B. pertussis producing lymphocytosis, and in increasing production of antibody of the reaginic type.

Various fractions of B. pertussis cells have been prepared and methods for studying their various biological activities have been developed. Dr. Bergman has adapted a method for studying the adjuvant activity of B. pertussis fractions that employs chicken gamma globulin (CGG) as an antigen. Sheep RBC coated with chicken anti-sheep RBC can be lysed by mouse anti-CGG, and thus one can enumerate the anti-CGG producing cells by the plaque technic. Furthermore, we found that as little as 5 gamma of SE given with 5 gamma of CGG induced the production of reaginic-like antibody to CGG. Hopefully we will develop a technic of enumerating the cells producing this antibody in addition to $7S\gamma_1$ and γM antibody producing cells.

4. Mechanism of action of HSF (Bergman). A great deal of effort has been spent to develop tests for the quantitation of catecholamines and adrenal steroids in mouse adrenal glands, as well as to determine adenyl cyclase in various mouse tissues.

An assay procedure utilizing two-step chromatography, internal standards of ^{14}C-epinephrine and ^{3}H-norepinephrine, and quantitation by fluorescence gave reasonably good results on assays of known standards of epinephrine and norepinephrine in the range of 0.1 to 10 µg/ml. Extraction of catecholamines from mouse adrenal glands and quantitation by the above technique gave results somewhat higher than other published values. Attempts to measure catecholamines in serum or plasma at physiological levels were not consistent. One major problem seemed to be an instability of the radiolabeled catecholamines. Repurification of ^{14}C-epinephrine corrected our overestimation of epinephrine in biological samples, but repurified ^{3}H-norepinephrine only partially reduced an overestimation of norepinephrine when we used ^{3}H-norepinephrine for an internal standard. The problem with stability of ^{3}H-norepinephrine seems to be a difficult one as indicated by the New England Nuclear Corporation.

Quantitation of mixtures of epinephrine and norepinephrine has depended on a differential fluorometric assay. More accurate analyses could be obtained if a good clear-cut separation of the two amines could be obtained by chromatography and each one assayed separately. In collaboration with Mr. Jerry Filz of Sorvall Laboratory and Dr. Ribi's group, we have developed a method which gave good separation of epinephrine and norepinephrine on silica gel in Centrichrome® glass columns, using ethyl acetate:methanol:acetic acid (60:35:5) as the solvent under N_2 gas pressure. Quantitation of the hormones in the eluate fractions is done by fluorescence. Thus far, the method has only been applied to standards, and whether it will work with biological preparations is yet to be tested.

We have obtained excellent separation of corticosterone and hydrocortisone on silica gel in stainless steel Sorvall columns utilizing standards in the 1 µg range. Smaller samples of steroid hormones placed on the column become dispersed so that quantitation is very difficult. In collaboration with Mr. Jerry Filz of Sorvall Laboratory we are attempting to design equipment that will give better resolution by keeping the samples from spreading into too much of the solvent volume.

The method of White and Zinsser (Analyt. Biochem. 41: 372, 1971) was used to assay for adenyl cyclase in mouse tissues and preparations of saline extract. By this method mouse spleen homogenates had quite low adenyl cyclase activity, kidney had a higher level, and the highest levels were measured in cerebral cortex homogenates. The presence of NaF in the incubation medium enhanced the adenyl cyclase activity. Preparations of SE from B. pertussis were found to have very low if any adenyl cyclase activity. One discrepancy between our work and that published is that we

were unable to enhance adenyl cyclase activity in the presence of catecholamines. The reason for our failure is unknown, but we anticipate trying some other assay procedures for the presence of adenyl cyclase activity.

5. Effect of HSF on slow virus infections (Munoz, Hadlow, and J. F. Bell). It has been found that 50 gamma of SE administered i.p. mixed with rabies virus induces a marked protective effect. Furthermore, an increase in the titers of antirabies virus antibodies in the blood of these mice was observed. Similar studies are presently underway in collaboration with Dr. Hadlow in mink receiving SE and Aleutian disease virus.

Significance to biomedical research and the program of the Institute:

More workers are now showing an interest in the biologically active substances found in B. pertussis cells. These active substances produce many intriguing effects, ranging from increased sensitivity to vasoactive amines and shocking agents to an increase in antibody production and susceptibility to autoimmune diseases. Various metabolic functions of the mouse are affected; thus, the sugar and fat metabolisms, the concentration of protein in plasma, and the response to epinephrine are markedly affected. Changes in the lymphoid tissue are also observed. One of the most interesting effects is the channeling of the immune response toward the production of greater amounts of γE-like antibody. Any one of these effects is of great significance to immunology. Our studies of necessity have been limited to only a few of these phenomena. Our main efforts have been to purify the HSF and explore its relationship to other activities of B. pertussis. The importance of isolating and identifying this active material is obvious.

Studies on the mechanism of action of HSF have already led to the speculation that similar mechanisms may operate in human asthma. The mechanism by which B. pertussis extracts increase autoimmune diseases in experimental animals may also have their counterparts in human autoimmune diseases.

The marked effect that endocrine glands may play in hypersensitivity reactions is one reason we have spent considerable effort to develop methods, applicable to mice, to quantitate the adrenal hormones and adenyl cyclase.

The protective antigen (PA) of B. pertussis is of practical importance. In the past we have not been able to separate HSF from PA and our current studies may furnish more information along these lines.

Proposed course of project:

During the next fiscal year we will study the relationship of HSF to the adjuvant activity, the protective antigen, the lymphocytic promoting substance and the agglutinogens of B. pertussis. The studies on the γE-like antibody by B. pertussis extracts and the further purification and characterization of HSF will be continued. The effect of B. pertussis extracts on the course of rabies and Aleutian disease will be continued.

Honors and Awards:

Dr. J. J. Munoz

Reviewer for the following journals: Journal of Infectious Diseases, Infection and Immunity, and Immunochemistry.

Invited to give a seminar on the histamine sensitizing factor of B. pertussis at the Scripps Clinic, LaJolla, California.

Publications:

Bergman, R. K. and Munoz, J.: Effects of epinephrine, norepinephrine and isoproterenol against histamine challenge in Bordetella pertussis-treated and beta-adrenergic blocked mice. Life Sci. 10: 561-568, 1971.

Munoz, J.: Protein toxins from Bordetella pertussis. In Kadis, S., Montie, T. C., and Ajl, S. J. (Eds.): Microbial Toxins, Bacterial Protein Toxins. New York, Academic Press, Inc., 1971, vol. 2A, pp. 271-300.

Serial No. NIAID-185
1. Rocky Mountain Laboratory
2. Serological Technology and Epidemiology Sections
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Agents of the psittacosis-lymphogranuloma-trachoma group (Chlamydia)

Previous Serial Number: Same (includes the former NIAID-182)

Principal Investigators: R. N. Philip and R. K. Gerloff

Other Investigators: Elizabeth Casper and L. A. Thomas

Cooperating Units: Dr. F. B. Gordon, Naval Medical Research Institute, Bethesda, Maryland
Dr. S. P. Wang, School of Public Health and Community Medicine, University of Washington, Seattle
Prof. Barrie R. Jones and Dr. John D. Treharne, Institute of Ophthalmology, Univ. of London, England
Dr. John L. Decker, NIAMD, NIH, Bethesda, Maryland
Dr. Robert A. Sylvester, Naval Medical Research Inst., Bethesda, Maryland

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	4 6/12			4 6/12
Professional:	2 4/12			2 4/12
Other:	2 2/12			2 2/12

Project Description:

Objectives:

The general purpose of this project is to investigate relationships among the chlamydiae, to clarify the epidemiology of chlamydial diseases and determine their total importance to human health, and to apply technological advances to the laboratory study of these organisms.

More specific objectives include: 1) improved methods for isolating chlamydiae from clinical specimens, 2) isolation and characterization of new strains, 3) more luxuriant growth in embryonated eggs and cell cultures, 4) simpler methods for purifying and quantitating elementary body suspensions, 5) more sensitive and specific diagnostic reagents and techniques, 6) antibody surveys to determine prevalence of chlamydial infection in specific human and animal populations, 7) study of chlamydial interrelationships and characteristics of possible taxonomic value, and 8) study in laboratory systems of the immunogenicity of whole organisms and fractions thereof.

Methods employed:

A wide variety of techniques were employed, most of which are standard for chlamydial, rickettsial and viral research or have been described in previous annual reports. Use of the micro-immunofluorescence (micro-IF) procedure for both serotyping of chlamydial strains and testing of human serums was emphasized over the past year.

Major findings:

1. Strain serotyping. Based on typing of isolates in several laboratories by micro-IF, the numbers of recognized serotypes of Chlamydia trachomatis are now 9 TRIC (types A-I) and 3 LGV (types I-III). We characterized 2 additional strains from the District of Columbia as type E. This brings the number of serotypes recovered in the Washington, D.C., area to 3 (TRIC, types E and I (ai) and LGV, type II). Direct typing of serums indicates that TRIC type F is probably also circulating in D.C.

Two isolates recovered by Dr. Gordon from the urethra and conjunctivae of a patient with Reiter's disease were similar to each other and were characterized as TRIC, type I (ai).

Serologic relationships of nonhuman isolates of chlamydiae, particularly ovine and bovine abortion strains, are also under investigation. We are encountering some difficulty in preparing potent mouse antiserums by procedures used with human strains and our findings are too preliminary for comment.

2. Testing of human serums. The usefulness of the micro-IF test for detecting and typing antibody in human serums was explored. Serums from various arthritis cases and patients attending a Washington, D.C., venereal disease (V.D.) clinic were tested against chlamydiae representing various TRIC and LGV serotypes known to infect the uro-genital tract of man. This work is nearing completion and the following are some of the more important observations.

The micro-IF test is a sensitive procedure in detecting antibody. While not quite as reactive as the RIP test it is some 8 times more sensitive than our standard CF test. Over 80% of the V.D. clinic serums were reactive in the micro-IF and RIP tests, whereas only 33% had CF antibodies.

Human serums are more broadly reactive than serums from immunized mice. Nevertheless, one-third of the V.D. clinic serums showed specificity against one or more test antigens ($\geq$4-fold differences in titers). Five of 8 persons from whom isolates were obtained and typed showed patterns of serologic specificity corresponding to the serotype of the isolate. Aside from LGV, we have not yet identified a particular clinical syndrome with infection by a given serotype.

Confirmation of specificity is being obtained in antibody absorption micro-IF tests using partially purified, hyperconcentrated yolk-sac suspensions of chlamydiae as absorbing antigens. Heterotypic antigens remove 90% or more of the heterologous reactivity from typable serums, but have little or no effect on titers of type-specific antibody. On the other hand, homotypic antigens remove antibody reactivity against all test antigens. That the heterologous antibodies are probably not chlamydial group antibodies was demonstrated by the rather surprising finding that absorption with a strain of Chlamydia psittaci (Stamp's ovine abortion agent) had little or no effect on antibody titers to any TRIC antigen. All human strains used as absorbing antigens removed heterologous antibody to about the same extent; this finding suggests the possibility of an antigen which is common to all human strains of C. trachomatis (i.e., a species-specific antigen).

The immunoglobulin classes of antibody in the V.D. clinic serums were determined with monospecific IgG, IgM, and IgA conjugated antiserums. In general, IgG patterns of reactivity paralleled those of undifferentiated antibody. This was not unexpected since conjugated antiserum to undifferentiated globulins probably consists largely of antibody to the IgG component. Approximately 20% of the serums showed IgM reactivity, which often was type specific (26 of 39 serums). Although substantial IgM antibody titers were found in several serums with little or no RIP or micro-IF IgG antibody, this generally was not the case and we could not conclude that IgM reactivity was usually indicative of recent infection. IgA antibodies were detected in 20% of the serums. Various combinations of antibody classes were observed without any striking pattern of association. Type specificity, particularly characteristic for IgM antibodies, was also found in the IgG and IgA classes. Where specificity could be demonstrated in more than one antibody class it was always to the same serotype.

3. Toxic effects of various chlamydiae on cell monolayers. Relatively little additional knowledge has been gained in this area over the past year, although considerable time has been spent. A paper reporting the results obtained earlier was rejected for publication because the journal editors wanted a check made for mycoplasma in cell cultures and chlamydial suspensions. Also they were reluctant to accept our findings about the severe effect of ultrasonicated normal L cell suspensions on mice inoculated i.v.

Throughout the study the mycoplasma issue had not been ignored, but we had been unable to isolate mycoplasma in two different media. When renewed attempts were later made with a third medium, a mycoplasma was found in the L cells. The necessity for repeating many of the toxicity experiments was obvious, however, after experience had been gained in detecting mycoplasmas and maintaining cell cultures free of them.

In summary, the characteristics previously described for the chlamydial toxic effect were again observed when mycoplasma-free systems were used. Likewise, ultrasonicated suspensions of mycoplasma-free cells representing several lines had the same severe effect previously observed. When cells were disrupted by one freeze-thaw cycle instead of sonication, the same results were obtained. This effect was not due to injection of particulate material, since supernates from high speed centrifugation affected mice in the same manner. The reaction in mice with uninfected control suspensions has made it difficult to compare precisely the toxicity of chlamydial suspensions in tissue culture with that in mice.

A previously unrecognized phenomenon associated with the chlamydial toxic effect is the marked acceleration in inclusion development that accompanies it. When monolayers are inoculated with particle suspension just concentrated enough to infect all cells, the developmental cycle of meningopneumonitis agent, for example, requires about 36 hrs and at 16 hrs inclusions are barely perceptible microscopically. As the concentration of inoculum is increased, inclusions develop more rapidly; when it reaches the toxic range, inclusions are 4 to 5 times larger than normal at 16 hrs. Under such conditions, the host cells rapidly deteriorate and lyse before inclusions reach maturity. The same phenomenon has been found to occur with strains of Chlamydia trachomatis.

4. Studies involving the RIP test. A group of 108 human serums submitted by Drs. Decker and Sylvester were subjected to RIP test for chlamydial antibody to explore possible relationships between past chlamydial infection and Reiter's syndrome. Statistical analysis by Dr. Decker did not show a definite relationship between titer of RIP antibody and Reiter's. However, a firm conclusion cannot be reached until the data are evaluated in light of the disease activity existing in the patients at the time of bleeding and the intervals between onset of symptoms and collection of serum samples. Such information is being collected.

On the basis of number and size of inclusions produced in L cell monolayers, certain strains of LGV (404) produce extremely heavy growth. This finding suggested use of strain 404 for preparing RIP antigen instead of the Cal 10 strain of meningopneumonitis agent used to date. Doing so would also enable a comparison of results with an antigen representing C. trachomatis rather than C. psittaci.

Results have been poor thus far. Under the conditions of labeling with P^{32}, many large inclusions consistently formed but were devoid of elementary bodies. Secondly, antigens prepared from labeled particles gave false positive reactions in serum dilutions below 1:512. Surface characteristics of the LGV particles seemed to cause them to adhere strongly but nonspecifically to globulin-antiglobulin precipitate.

Significance to biomedical research and the program of the Institute:

A major handicap to our understanding of chlamydial infections has been the lack of suitable serologic techniques for identifying infection and the nature of infecting organisms. The micro-IF test appears to meet some of the requirements. It is sensitive and also possesses the unique characteristic that it can differentiate antibody responses, at least to those serotypes of C. trachomatis which are markedly dissimilar antigenically. In the past we have had to rely on laborious, insensitive isolation procedures to identify the nature of infecting organisms. From the results of serologic surveys with the RIP test, now confirmed by micro-IF, the evidence is quite convincing that venereally transmitted chlamydial infections are frequent among sexually indiscriminate persons. However, the role of these organisms in diseases of the urogenital tract is still undefined. The micro-IF test should be a useful technique for clarifying this role.

Chlamydial toxic effects in cell cultures are important mainly from the laboratory point of view. They must be considered when inoculated monolayers are centrifuged to enhance infection; otherwise yields of particles can be very low. Neutralization of the effect shows some promise as a tool in taxonomy.

The RIP test for chlamydial antibody continues to attract attention as a very sensitive detector of antibody and, to our knowledge, is not being performed elsewhere. Thus requests continue to come from other investigators for testing of serums in connection with clinical and epidemiological studies.

Proposed course of project:

Application of the micro-IF test to field study will be emphasized as soon as the parameters of the test are adequately defined. A major disease problem in this country and Great Britain is nonspecific urethritis.

Chlamydiae have been suspect as causal agents in some instances. Negotiations are under way with the Great Falls City Health Department to obtain specimens from nonspecific urethritis and other V.D. cases for isolation attempts and serologic study. The characteristics of antibody at the site of infection will be investigated by micro-IF to determine whether the serotype specificity of locally produced antibody would be of diagnostic value.

Serotyping of animal strains of C. psittaci will continue as time permits. The only convincing evidence of protection by a chlamydial vaccine has been obtained with one prepared from the ovine abortion agent. Epizootic bovine abortion, as well as ovine abortion, is a significant economic problem in animal husbandry. A method for serotyping would be useful in determining the homogeneity and the antigenic relationships among chlamydial isolates from these two diseases, and in providing an indicator for the practicability of vaccine prophylaxis.

A greater proportion of time will be devoted to the RIP test during the coming year. One project, in cooperation with Dr. Peter Reeve in London, is being arranged for testing of serums from cases of nonspecific urethritis. In addition, more basic knowledge will be sought regarding the test itself. Different strains of C. trachomatis and C. psittaci will be tested for suitability for antigens. The significance of low titers will be evaluated, and antiglobulin prepared against highly purified gamma globulins will be tried to increase specificity of the test. The classes of immunoglobulin active in the RIP test also will be investigated.

Publications:

Gerloff, R. K., Ritter, D. B., and Watson, R. O.: Counting chlamydial particles by negative staining with Congo red. J. Infect. Dis. 123: 429-432, 1971.

Waldhalm, D. G., Frank, F. W., Meinershagen, W. A., Philip, R. N., and Thomas, L. A.: Lambing performance of ewes inoculated with Chlamydia sp. before and after breeding. Amer. J. Vet. Res. 32: 809-811, 1971.

Philip, R. N. and Whitlock, J. J.: Immunologic studies of lymphogranuloma venereum and mouse-adapted TRIC chlamydiae in mice. In Nichols, R. L. (Ed.): Trachoma and Related Disorders Caused by Chlamydial Agents. Proceedings of a Symposium, Boston, Aug. 17-20, 1970. Amsterdam, Excerpta Medica International Congress Series No. 223, 1971, pp. 124-132.

Miller, L. T. and Gerloff, R. K.: Reactions of human sera to chlamydia and avian mycoplasma. Avian Dis. 15: 789-797, 1971.

Philip, R. N., Hill, D. A., Greaves, A. B., Gordon, F. B., Quan, A. L., Gerloff, R. K., and Thomas, L. A.: Study of chlamydiae in patients with lymphogranuloma venereum and urethritis attending a venereal diseases clinic. Brit. J. Vener. Dis. 47: 114-121, 1971.

Serial No. NIAID-186
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: The biology of microbial agents in arthropods

Previous Serial Number: Same

Principal Investigators: W. Burgdorfer and L. P. Brinton

Other Investigators: R. A. Ormsbee, M. G. Peacock, L. A. Thomas, and D. A. Wike

Cooperating Units: NAMRU-3, Cairo, Egypt
Major D. L. Huxsoll, Walter Reed Army Institute of Research, Division of Veterinary Medicine

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 10/12		2/12	4
Professional:	1 6/12		2/12	1 8/12
Other:	2 4/12			2 4/12

Project Description:

Objectives:

To study dynamics of viral, rickettsial and bacterial infections in arthropod vectors and the interactions between microorganisms and arthropod host, as reflected by changes in morphology, histology and fine structure of arthropod tissues. Specifically investigated are 1) development of pathogens within growth-supporting cells, 2) cellular response(s) to infection, 3) effects on physiologic characteristics resulting from pathogen-arthropod association, and 4) factors supporting or inhibiting development and suvival of infectious agents.

Research during the past year included 1) electron microscopic studies related to (a) spermiogenesis and sperm behavior in the female tick's genital system, (b) transovarian transmission of rickettsial agents, (c) interactions between tick hemocytes and pathogenic rickettsiae, (d) structure of nonpathogenic rickettsialike organisms of ticks, 2) final attempts to infect ixodid ticks with Rickettsia prowazeki, 3) vector relationship of R. canada with emphasis on the lone-star tick, Amblyomma americanum,and the rat flea, Xenopsylla cheopis, 4) behavioral studies of virulent spotted fever rickettsiae in Dermacentor variabilis and A. americanum, 5) application of primary chick embryo tissue culture to isolation and characterization of rickettsia and rickettsialike organisms from ticks, and 6) studies related to development of Ehrlichia canis in Rhipicephalus sanguineus.

Methods employed:

In addition to routine techniques of medical entomology, bacteriology, and virology, fluorescent antibody procedures are applied to studies of viral, bacterial, and rickettsial infections in arthropods. Electron microscopy is used to characterize subcellular structures of normal and infected arthropod tissues and to determine morphology of microorganisms during intracellular development.

Major findings:

1. Upon completion of spermiogenesis in the seminal receptacle of female ticks and ensuing rupture of the endospermatophoric wall, large numbers of aflagellate sperm migrate up one or both oviducts. During passage through the paired oviducts, some sperm, in close proximity to the oviducal wall, tend to penetrate oviducal epithelial cells. This phenomenon is further evident in the ovary where sperm partially invade the lumenal epithelial cells. These cells respond to intracellular invasion by formation and deposition of a finely granular capsular material around the invading portion of sperm cells. Such sperm cell behavior is interpreted to be a physiochemical taxis of sperm seeking gravid oocytes.

In studies of the dynamics of "transovarian transmission" of spotted fever group rickettsiae, we found, by electron microscopy, that rickettsiae in unfed nymphal ticks are present primarily in the epithelial cells of the ovarian external sheath and rarely in oogonial cells. Invasion of the latter probably occurs during nymphal feeding and subsequent molting processes. In newly molted females, rickettsiae are consistently seen in the ooplasm of oocytes where they undergo intense multiplication and invasion of cell nuclei during subsequent tick feeding.

Subcellular studies on interactions between tick hemocytes and pathogenic rickettsiae revealed rather uniform reactions of blood cells towards R. rickettsi, R. montana and R. canada. Of various cell types, spherular hemocytes and plasmatocytes most frequently contained rickettsiae acquired by direct invasion or phagocytosis. In spherule cells, rickettsial cell walls are in close association with their "protoplast", which contain distinct ribosomes in a cytoplasmic matrix interspersed with a fine reticulum characteristic of developing rickettsiae. In plasmatocytes, on the other hand, rickettsial cell walls appear expanded or swollen and well separated from the "protoplast" whose cytoplasmic matrix possesses poorly defined ribosomes and no discernible reticulum.

Studies of developmental fine structure of nonpathogenic rickettsia-like organisms in D. andersoni revealed three morphologic forms: filamentous or threadlike, chain, and short rod or bacillary forms. Fine structure of cell walls and plasma membranes of all forms are typically tri-layered as in pathogenic rickettsiae. Cytoplasmic matrix contain ribosomes in a finely filamentous reticulum.

2. Although adult D. andersoni and A. variegatum could readily be infected by intracelomic inoculation with infectious yolk-sac suspensions containing the Cairo-III strain of R. prowazeki, all attempts failed to initiate generalized and prolonged infections by feeding larval and/or nymphal D. andersoni, A. variegatum, A. cohaerens, Rhipicephalus sanguineus, R. evertsi and Hyalomma dromedarii on rickettsemic guinea pigs and/or voles (Microtus pennsylvanicus). Rickettsiae were ingested by all species except A. cohaerens, and in some instances were maintained transstadially into the next developmental stage. However, such infections were limited to the tissues of the midgut, and of temporary nature only. Prolonged existence of rickettsial infection, for at least 84 days, was demonstrated in only one instance. In this case, suspensions of freshly engorged nymphal D. andersoni that initially had been infected as larvae produced specific agglutinins in voles. However, these rickettsiae did not survive the molting processes to adults.

3. Further evidence of an adverse effect of R. canada on the biologic development of ticks was obtained through studies of the second filial generation of ovarially infected D. variabilis. Massive rickettsial growth, particularly during feeding of F_2 larvae, produced considerable disruption of cellular structures and killed the majority of ticks. On the other hand, the lone-star tick, A. americanum, appears to tolerate R. canada, which was passed transstadially and transovarially in a fashion similar to that of spotted fever rickettsiae. Although there is some evidence of mortality among larvae before and after engorgement, several lines of infected ticks could be maintained through two complete filial generations.

Attempts to infect the rat flea, Xenopsylla cheopis, by feeding specimens once or even repeatedly on R. canada infected meadow voles failed. However, infection could be established in about 50 percent of fleas by feeding through mouse-skin membranes upon a 10 percent suspension of infectious yolk sac in defibrinated rabbit blood. Daily microscopic examination of tissue smears revealed particulate antigen in the midgut for the first 15 days, and thereafter, increasing numbers of morphologically typical rickettsiae. Rickettsial growth was generally mild and limited to the epithelial lining of the midgut. Transmission experiments at 5-day intervals after the fleas' infectious feedings have been uniformly negative. Similarly, R. canada could not be demonstrated either microscopically or by animal inoculation in the fecal material of infected fleas.

4. Virulent strains of R. rickettsi isolated from the Rocky Mountain wood tick, D. andersoni, were shown to have an injurious effect on the biologic development of D. variabilis and A. americanum as indicated by mortality rates as high as 100 percent among engorged adults that had been infected as larvae or nymphs. Surviving females consistently produced small batches of eggs that failed to develop.

5. Primary chick embryo tissue cultures were successfully applied to isolation of spotted fever group rickettsiae from the hemolymph of infected ticks. Experiments are in progress using this technique for isolation and cultivation of rickettsialike agents nonpathogenic for laboratory animals (see Annual Report 1970/71). Preliminary results indicate that these organisms, considered to be related to pathogenic rickettsiae, do form plaques similar to those produced by pathogenic rickettsiae.

6. Several attempts to demonstrate by conventional microscopy Ehrlichia canis in adult Rhipicephalus sanguineus that had been fed (at Walter Reed Research Institute) as larvae or nymphs on dogs infected with tropical canine pancytopenia gave negative or inconclusive results.

Significance to biomedical research and the program of the Institute:

It has long been postulated that sexual transmission of spotted fever rickettsiae may occur by the transfer of infected tick sperm, and it is speculated that vectorial capacity of ticks and other hematophagous arthropods is linked to genetic markers and chromosome maps. Research along these lines, like other investigations related to vector-parasite relationships, is meaningless unless a thorough knowledge of developmental morphology, histology and fine structure of vector tissues as well as of the various biologic and physiologic processes occurring during vector development is obtained. Gross histologic and electron microscopic aspects of ovarian maturation (reported last year) and of spermatid-sperm behavior in ticks are part of our efforts to fulfill these requirements.

Minimum dose requirement for successful infection of ixodid ticks with R. prowazeki has been shown to be considerably higher than that provided by concentrations of rickettsiae circulating in the blood of infected laboratory animals tested so far. Until animals are found in nature that meet this minimum dose requirement, incrimination of ticks as vectors of epidemic typhus does not appear justified.

Amblyomma americanum is a potential vector of R. canada by virtue of its capability to maintain this agent transstadially as well as trans-ovarially. This tick, unlike D. andersoni and D. variabilis, is not adversely affected by R. canada and represents the dominant species in areas where several human cases of presumed R. canada infections did occur. The rat flea, Xenopsylla cheopis, the vector of R. typhi, does not appear to be involved inthe ecology of R. canada.

High mortality rates among D. variabilis, A. americanum and occasionally also among D. andersoni infected with virulent R. rickettsi suggest that the relationship between this agent and its tick vectors, generally thought to be purely symbiotic in nature, may occasionally become a parasitic one.

Primary chick embryo tissue culture (see also NIAID-171) appears to be an ideal tool for isolating pathogenic as well as nonpathogenic rickettsial agents from naturally infected ticks. Formation of plaques similar to those formed by R. rickettsi by the nonpathogenic, long rickettsial organisms present in a large percentage of ticks strongly supports previous findings (by fluorescent antibody staining techniques), suggesting a close relationship of these organisms to spotted fever group rickettsiae.

Failure to microscopically detect Ehrlichia canis in infected R. sanguineus indicates a developmental cycle of this agent in ticks different from that of other rickettsial agents.

Proposed course of project:

Lack of technical assistance for more than half a year in the electron microscopy unit resulted in a considerable slow-down of research activities. Thus, the study related to development and subcellular relationship of Colorado tick fever virus in D. andersoni did not progress beyond its initial stage; it will receive priority during the coming year. Once technical help becomes available and is sufficiently trained, a long-term project will be undertaken to establish subcellular structures of tick salivary gland. This project will be in conjunction with studies concerning physiochemical characterization of tick saliva and development of viral and rickettsial agents in salivary gland tissues.

Fluorescein isothiocyanate-labeled conjugates against Ehrlichia canis have been prepared and will be applied to future studies of this agent in ticks. Additional attempts are presently being made at Walter Reed Army Institute of Research to infect R. sanguineus and also D. variabilis with this agent by feeding ticks on parasitemic dogs.

Presence of spotted fever group rickettsiae in wild-caught Ixodes scapularis suggests involvement of this species of tick in the ecology of R. rickettsi. An experimental study is planned to evaluate the vector potentiality of this tick.

There is little information on the vector-parasite relationship of Ixodes holocyclus and R. australis, the etiologic agent of Queensland tick typhus. With cooperation of Drs. Wharton and Stone, CSIRO, Queensland, Australia, a study is programmed to provide this long-sought knowledge. Preparations for establishing at RML a productive colony of I. holocyclus are presently under way.

Usefulness of primary chick embryo tissue cultures for isolating rickettsial agents from ticks collected in nature will be evaluated during the forthcoming tick season in western Montana.

Honors and Awards:

Dr. Willy Burgdorfer

Reappointed Associate Member of the Commission on Rickettsial Diseases, Armed Forces Epidemiological Board.

Appointed Consultant to University of Maryland Medical School (Dr. Charles L. Wisseman, Jr.) on contract NR-136-889 related to tickborne pathogens and extra-human typhus infections.

Continued to serve as Faculty Affiliate for the Department of Zoology, University of Montana, Missoula.

By request reviewed research manuscripts for Journal of Epidemiology, Journal of Parasitology, and Journal of Medical Entomology.

Dr. L. P. Brinton

Invited to serve as discussant for the Symposium on Reproduction of Arthropods of Medical and Veterinary Importance, Fourteenth International Congress of Entomology, Canberra, Australia, Aug. 22-30, 1972.

Publications:

Brinton, L. P. and Oliver, J. H., Jr.: Gross anatomical, histological, and cytological aspects of ovarian development in Dermacentor andersoni Stiles (Acari: Ixodidae). J. Parasit. 57: 708-719, 1971.

Brinton, L. P. and Oliver, J. H., Jr.: Fine structure of oogonial and oocyte development in Dermacentor andersoni Stiles (Acari: Ixodidae). J. Parasit. 57: 720-747, 1971.

Brinton, L. P. and Burgdorfer, W.: Fine structure of normal hemocytes in Dermacentor andersoni Stiles (Acari: Ixodidae). J. Parasit. 57: 1110-1127, 1971.

Brinton, L. P.: Ovarian intercellular bridges in Dermacentor andersoni Stiles (Acari: Ixodidae). Tissue and Cell 3: 615-622, 1971.

Serial No. NIAID-187
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

PHS-NIH
Individual Project Report
July 1, 1971 through June 30, 1972

Project Title: Host-ectoparasite relationships

Previous Serial Number: Same

Principal Investigator: C. M. Clifford

Other Investigators: J. F. Bell, B. M. Hestekin, J. E. Keirans, and C. E. Yunker

Cooperating Units: Dr. W. A. Nelson, Canada Department of Agriculture Research Station, Lethbridge, Alberta

Man Years:	NIAID Lab Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	1 9/12			1 9/12
Professional:	10/12			10/12
Other:	11/12			11/12

Project Description:

Objectives:

Studies are directed toward classification of certain phases of host/arthropod relationships. Emphasis is centered on identifying the mechanisms by which animals become resistant to feeding of arthropods and factors governing responses of parasitized animals to infection with various microorganisms.

Methods employed:

The host-parasite system developed at the RML whereby measurable and predictable populations of lice can be developed on various strains of mice by restriction of self-grooming is extensively used. At times other laboratory animals are utilized and a variety of microbiologic, immunologic and entomologic techniques are employed.

Major findings:

The RML strain of White Swiss mice was used in all the following experiments. A-2 refers to mice in which grooming is restricted by amputation of the hind limbs when they are less than 1 day old.

1. LD_{50} determinations. In many phases of current infestations it was necessary to determine the precise number of lice that would cause the death of 50% of the mice of a given age. When A-2 male and female mice of 14 gm average weight were seeded at 3 levels—5, 15, and 45 mg/mouse, the LD_{50} at 1 week after seeding was 45 mg lice/mouse whereas at 4 weeks it was 34 mg/mouse. Other experiments have demonstrated that LD_{50} is directly proportional—weight of mouse versus the concentration of lice seeded.

2. Permeability studies of blood vessels in the skin in heavily parasitized areas. A group of mice was infested with lice and scored at weekly intervals. At each interval mice with known scores were inoculated IV with 0.5% Evans Blue 0.2 ml per mouse and killed 30 minutes later. Increased permeability was noted through the 6th week of infestation. Then a gradual decrease ensued to the 14th week.

3. Induction of tolerance. Newborn mice were exposed to heavy louse populations within 24 hrs of birth for a period of 2 hrs. Exposure was repeated at 7 and 14 days of age, for periods of 18 and 24 hrs, respectively. Mice were treated with Kwell after each exposure. Control mice (no exposure to lice) were handled in an identical pattern as exposed mice throughout experiment. All mice were weaned at 21 days of age and each was seeded with 8 mg of lice per mouse (20 mice/group). No significant difference in scoring was noted in exposed versus control mice; however, 70% of the exposed mice died versus 35% of the controls.

In another tolerance experiment ground lice were injected on corresponding days instead of exposing the mice to feeding lice. Through the 10th week, differences between the injected mice and controls were not significant with respect to scoring or mortalities.

4. Hematocrit studies. An indirect correlation was demonstrated between louse scoring and hematocrit values, when A-2 mice were seeded with 5 mg of lice per mouse. Scores of 40 extended from the 2nd through the 4th week while percent packed cells declined to 31% by the 4th week. By the 8th week, scorings averaged 15 and percent packed cells returned to a normal value of 51%, and remained at this level through the following weeks.

5. Effect of immunosuppressants on louse infestation. Effects in mice of antithymocyte serum, cyclophosphamide, glass powder and endotoxin upon acquired resistance to lice were studied. A-2 mice that received 0.25 ml rabbit antimouse thymocyte serum IP on days 1, 7, 35, and 56 after seeding had slightly higher louse scores between the 8th and 14th weeks

than controls, but the difference may not be significant. Those that received 4 mg of cyclophosphamide IP on days 1, 7, and 18 after seeding had higher scores than controls between the 6th and 14th week, and mortality was 60 to 80% greater than controls. Glass powder and endotoxin did not affect the course of the infestation.

6. Effects of histamine-sensitizing factor (HSF) (an extract from Bordetella pertussis) on louse populations and mortalities. Four groups of mice were injected with 40 µg of HSF per mouse. Three of these groups were then seeded one day later with either 2.5, 10, or 40 mg of lice per mouse; one group was maintained as controls. Observations through the 8th week after exposure indicate that a single dose of HSF did not affect acquired resistance.

Significance to biomedical research and the program of the Institute:

The aim of this project is an understanding of the mechanism whereby mice become resistant to the feeding of lice. This study has been possible because of the mouse-louse experimental system developed at RML. Information from the study could aid in development of methods for protecting man and animals against deleterious effects of the feeding of arthropods. The mouse-louse system offers a unique opportunity to study the development of resistance to arthropod feeding since the experiments can be conducted at a relatively low cost.

The need for work on various aspects of the arthropod-host relationship is becoming increasingly evident. This fact was amply brought out when the host response to arthropod bites was selected as one of two topics to be discussed at a symposium held in Hamilton, Montana, on September 15, 1971, sponsored jointly by the Tropical Medicine and Parasitology Study Section, Division of Research Grants, NIH, and the Rocky Mountain Laboratory staff. A summary of the highlights of this meeting will be published soon in Science.

Proposed course of project:

Work during the next year will continue on attempts to understand more about the mechanism that allows mice to become resistant to lice. The work on passive cell transfer will be repeated using inbred strains of mice.

Publications:

Nelson, W. A., Clifford, C. M., Bell, J. F., and Hestekin, B.: Polyplax serrata: Histopathology of the skin of louse-infested mice. Exp. Parasit. 31: 194-202, 1972.

Serial No. NIAID-188
1. Rocky Mountain Laboratory
2. Medical Entomology and Acarology Section
3. Hamilton, Montana

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Individual Project Report
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Project Title: Vector-pathogen relationships

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Other Investigators: C. M. Clifford, J. Cory, J. E. Keirans, R. Parker (PHS Postdoctoral Fellow), and E. Ribi

Cooperating Units: Dr. M. N. Kaiser, NAMRU-3, Cairo, Egypt

Man Years:	NIAID Lab. Staff	Other NIH Personnel Assigned to Project	Guest Workers	Total
Total:	3 5/12		1	4 5/12
Professional:	1 4/12		1	2 4/12
Other:	2 1/12			2 1/12

Contract Support:

Contract Number	Project Officer (NIAID)	Contractor	Contractor's Project Director	Annual Funding Level
PL-480 03-016-1	C. E. Yunker	NAMRU-3 Cairo, Egypt	M. N. Kaiser	$30,145

Project Description:

Objectives:

1) To investigate the host-range, cellular relationships and growth dynamics in arthropods or their cells of pathogenic agents transmissible

by vectors among man or animals, 2) to develop systems of vector- or vector-related-tissue and cell culture and to apply these systems to the study of arthropod-borne disease and vector-pathogen relationships, 3) to define the microenvironment to which pathogenic microorganisms are subjected when ingested by tick vectors, 4) to provide basic biochemical information necessary for the progress of developmental tick tissue culture studies.

Methods employed:

Disease agents of primary interest are arthropod-borne viruses and, to a lesser extent, rickettsias, which are transmitted by Acarina and Diptera. Thus, conventional basic techniques in laboratory arbovirology and experimental acarology and entomology are employed. A major effort involves development and use of arthropod tissue and cell cultures. Standard and specially devised biochemical and physiological techniques are used to determine the nature of tick body fluids. Resultant findings are employed in the preparation of tick tissue culture medium in efforts to improve the growth supporting characteristics of the medium.

Major findings:

1. Significant progress was made in our continuing efforts to produce visible evidence of infection of arthropod cell systems with arboviruses. A plaquing technique was developed that permits assay of certain arboviruses in Aedes albopictus cell monolayers. Sixteen of 58 viruses produced clearly defined plaques ranging in diameter from 1 to 7 mm, depending on the virus involved. Plaque numbers were proportionate to dilutions inoculated, within the range of those produced in Vero cells by the same dilutions, and reducible by prior neutralization of the virus with immune serum. Six nonarboviruses and 4 rickettsias failed to produce plaques. Plaquing viruses were: Chikungunya, West Nile, dengue 1, 2, 3 and 4, yellow fever (YF), Japanese B encephalitis, St. Louis encephalitis, Murray Valley encephalitis, Israel turkey meningoencephalitis, Chitoor, Germiston, Guaroa, and Indiana vesicular stomatitis. All of these are known or presumed to be insect-borne and 10 are members of Casals' serological group B (flaviviruses). Twenty-six tick-borne viruses, mostly orbiviruses of the Kemerovo group, but including 6 flaviviruses, failed to produce plaques, but one Kemerovo-group virus, Tribec, did so consistently. Because all but 6 viruses that plaqued will grow in fluid cultures of Ae. albopictus cells without obvious cytopathogenic effect (CPE) 8 attempts were made to induce plaque formation by inoculating these cells with Colorado tick fever (CTF) virus, but all failed. This is one of 3 tick-borne orbiviruses known to multiply in the Ae. albopictus cell line.

2. Growth dynamics of CTF virus in arthropod cells were studied under various conditions of temperature and cultivation. The virus grows well in Ae. albopictus cells at temperatures from 27° to 37°C. At 27°C maximum yield was 4.5 logs, but 19 days were needed to produce this. At 35° and 37°C, 4.7 and 3.2 logs of virus, respectively, were seen on days 15 and 12. This time-temperature response is not linear, however, because a yield of 5.5 logs of virus could be produced only 2 days after inoculation when cultures were incubated at 32°C. In another experiment, Dermacentor andersoni primary explants infected with CTF virus were shown to yield most virus (6.5 logs) on day 7, if incubated at 27°C and cultivated between the curved glass of the culture tube and an overlying cover glass. Omission of serum in the culture medium did not affect virus yield.

3. We continued to compare arbovirus growth in insect cell and tick tissue culture systems. Emphasis was on viruses other than flaviviruses. Eight tick-borne viruses, 5 mosquito-borne viruses and one virus not arthropod-borne were inoculated into 3 insect cell lines and 2 tick tissue culture systems. Singh's Ae. albopictus cells supported the growth of the culicine-borne viruses, Chitoor, California, Trivitattus, and Turlock virus and, in addition, of 2 tick-borne members of the Kemerovo group, Tribec and Sixgun City viruses. They failed to support growth of the tick-borne viruses, Ganjam, Punta Salinas, Soldado, Green Kure, and Sapphire I, or of Myxoma virus. Singh's Ae. aegypti cells readily supported growth of Chitoor, Tribeć, California, and Trivittatus viruses but none of the others. Hsu's Culex quinquefasciatus cells were refractory to all of the above.

Primary cultures of explanted D. andersoni viscera or of D. andersoni hemocytes supported growth of the tick-borne viruses, Tribec, Soldado, and Punta Salinas and, whereas the viscera cultures also supported growth of Quaranfil, Yaquina Head, and Sixgun City viruses, hemocyte cultures failed to do so. In addition, neither system permitted growth of any of the above mosquito-borne viruses or of the tick-borne entities, Ganjam, Green Kure, Sapphire I or Chenuda viruses.

These results, with previous findings and the plaquing results reported above, support a hypothesis of specificity of cultured insect cells for insect-borne flaviviruses and of cultured tick cells for tick-borne flaviviruses. With regard to orbiviruses, particularly of the Kemerovo group, specificity is less marked and viruses may cross vector-class lines.

4. The serum-free substrain of Singh's Ae. albopictus cells (which we described in a previous report), was compared with the parent strain of serum-dependent cells with regard to growth characteristics and virus supporting ability. The former, now in its 73rd serum-free passage grows as fast as the serum-dependent strain (population doubling time 2.0 days).

When these strains were inoculated with 3 different arboviruses various growth patterns were seen: Chikungunya (an alphavirus) grew equally well in either system, CTF (an orbivirus) grew markedly better in the serum-free system, Chitoor (unclassified) could be grown only in the serum-dependent cells. Interpretation of these results must await further tests.

5. In previous experiments on the growth of Tuleniy virus in arthropod cell cultures, prolonged survival of virus was noted in cell-free growth medium used for Antheraea cells. Therefore, the stability of various arboviruses in this medium was compared with that in conventional diluents and in other tissue culture media. Antheraea cell culture medium was significantly best as a virus stabilizer at room temperature for at least 9 of 21 viruses in 3 groups. Further work is in progress.

6. By treating Ae. albopictus cells with mitomycin cell division was halted without loss of cell viability. Virus supporting ability of these cells was then tested. Both YF and CTF viruses replicated in the mitomycin treated cultures to the same extent as in untreated cultures. In addition, YF virus caused plaques in treated Ae. albopictus cells.

7. The tick hemocyte cultivation technique previously developed for the hard tick D. andersoni was adapted for use with the soft tick Argas persicus. As of now these hemocytes are still viable in vitro (41 days after explantation), and cultures inoculated with Quaranfil virus, which is transmitted by soft ticks, continue to yield virus in the medium.

In other developmental aspects of tick tissue culture, attempts were made to galvanically or chemically stimulate cell division. Primary tick tissue explants, and the Antheraea and Ae. albopictus cell lines were continually subjected to currents of 10-50 microamperes while in stationary or spinner cultures. In each cell-system and at all amperages no evidence of increased growth rate was seen; in fact, many cultures thus treated showed a retarded rate of growth. Similar attempts to stimulate growth were made with ecdysone, a molting hormone of moth origin. Growth rate of Antheraea (moth) cells was slightly increased, but that of other systems was either unaffected or retarded.

8. Analytical studies of arthropod tissue culture media were conducted. Changes in protein, amino acid and carbohydrate constituents before and after exposure to arthropod cells were observed. Significant changes were noted in sugars, whose consumption was in direct proportion to cell growth. Centrifugal chromatography, recently developed at this laboratory, was used for the separation of sugars in arthropod hemolymphs and tissue culture media. This method, unlike the older, thin-layer chromatographic method, proved readily applicable to quantitation of sugars.

9. As in the past, much effort was devoted to monitoring the purity and species-identity of our cell lines and the quality of our commercially obtained tissue culture media and supplements. We continued to provide outside investigators with insect cell lines, information on cultivation of tick and insect cells, and training in handling and plaquing of invertebrate cell cultures.

10. Considerable progress was made in tick biochemistry and physiology studies under PL-480 Agreement No. 03-016-1. This included:

a. Completion of a quantitatively and qualitatively descriptive study of tick hemocytes during different physiological stages.

b. Demonstration that both hard and soft female ticks have a metabolically controlled, adaptive spiracular closing mechanism in the unfed state. This mechanism is nonfunctional in recently engorged females.

c. The metabolism of glucose, previously determined to be the principal sugar of ticks, was studied. In Hyalomma ticks it was shown, by comparisons of gut content and hemolymph with host blood, that glucose concentrations increase during feeding, probably by water elimination through salivation. Oral secretions of these ticks yielded only trace amounts of glucose. In Argas ticks, in which coxal fluid excretion rather than salivation is the principal mechanism for elimination of excess water from blood meals, glucose concentrations decrease from host-blood to gut-content to hemolymph. Here coxal fluid contained significant amounts of glucose. Glucose levels in Hyalomma decreased considerably during oocyte development and in correlation with high O_2 consumption levels.

d. Changes in free amino acid (FAA) and protein concentrations were studied in 2 Argas species. The gut-content and hemolymph FAA concentrations were similar in both species, lowest in the freshly engorged state, and increased with digestion and hemolymph reduction by water loss. Protein concentrations followed a similar curve but differed between species. Unlike FAA concentrations, protein levels were much lower in the Argas species than in Hyalommas previously tested. Protein and FAA concentrations in coxal fluids of Argas were 10% of that of hemolymph. In this regard, then, this fluid is essentially a dilute hemolymph. Obviously the coxal organ limits loss of these substances and regulates hemolymph composition.

e. Cell-free lipid components of ticks in various physiological states were studied both qualitatively and quantitatively using gas-liquid chromatography. Components included phospholipids, free sterols, free

fatty acids, mono-, di- and triglycerides and sterol esters. The fate of some was traced from gut content (as ingested blood) to eggs. Results suggest that gut lipids are absorbed in the ester form whereas hemolymph lipids release their fatty acid moiety for energy production and utilization in metamorphosis and molting. In addition, hemolymph fatty acids donate immediate energy in the fast anabolic reactions of oogenesis and development.

f. Guanine was identified as the sole purine component of Argas excreta and the major nitrogenous end product of each postembryonic stage and all feeding periods. In ixodid excreta an undescribed purine was found, which constituted up to 15% of the white nitrogenous excreta.

g. Rates of hemolysis of host erythrocytes were determined for 2 Argas species. Fastest rates were associated with natural hosts. These rates could not be correlated with erythrocyte osmotic fragility. In both species, in contrast to bloodsucking insects, proteinase levels increased markedly immediately after engorgement, indicating possible presence of an inactive precursor enzyme in the unfed tick.

These and many additional findings are reported in 17 research papers published or in press during the last year. In addition, 2 Master of Science theses based on work performed under this contract have been accepted by Cairo University Faculty of Science.

Significance to bio-medical research and the program of the Institute:

Because production of CPE by arboviruses in vector cells is rare and interference techniques are lacking, the development of a reproducible plaquing technique for many of these pathogens in mosquito-cell lines is especially gratifying. For the first time growth of viruses may be quantitatively assayed in these cells without resorting to a secondary assay system. This holds special significance for epidemiologic studies of certain viral diseases. For example, workers at CDC have found Ae. albopictus cells to be equal or superior to mammalian systems for primary isolation of dengue 2 viruses from human bloods and mosquito pools. However, identification required the use of a secondary system, monkey kidney tissue culture. The impractical need for more than one tissue culture system, when dealing with large numbers of specimens, could be avoided by performing plaque-reduction neutralization tests with the mosquito-cell line.

Results of arbovirus-arthropod cell culture specificities are useful when related to the emergining classification of arboviruses. Taxa of this artificial group are now becoming objectively defined by properties of the virion. The specificities detected here will provide additional insight into relatedness and evolution of these vector-dependent pathogens and facilitate their identification and recognition of vectors.

Argasid or soft ticks are vectors of a large number of arboviruses, some of significance to human medicine. The development of techniques for cultivation of their hemocytes increases the number of tools available for studies of virus-vector relationships at the cellular level.

Analytical studies of the biochemical composition and metabolic activities of tick- and tick-tissue culture fluids provide knowledge of factors necessary for survival, propagation and transmission of pathogens and aids in efforts to develop continuous cultures of tick cells.

Proposed course of project:

Arthropod tissue culture is an embryonic field which requires much effort to develop its potential value for studies of infectious disease. Efforts to characterize arbovirus growth in these systems and to augment their utility will continue. Emphasis will be placed on the expression of pathogenicity. For example, mosquito cells whose mitotic capabilities have been arrested will be used in an attempt to circumvent the disparate growth rates between pathogen and arthropod cell, thus leading to an expression of CPE. This will be particularly applicable in the case of rickettsias, which are rapidly "overgrown" by insect cells in vitro. Studies will be undertaken to determine how plaques can be formed by viruses that cause no obvious CPE in fluid monolayers. Continued support for the PL-480 project on tick biochemistry and physiology is anticipated and efforts here will be devoted mainly to D. andersoni analyses. The search for substances or means to stimulate division of tick cells will be continued. For example, attempts will be made to extract hormonal substances from ticks that will have the same effect on growth of tick cells that ecdysone has on Antheraea cells. Also, tick cells will be cultivated on "feeder layers" of mitotically inhibited mosquito cells.

Honors and Awards:

Dr. C. E. Yunker

Invited to present "Highlights of Medical Entomology" at the 1972 meeting of the Entomological Society of America.

Nominated for Annual Acarology Award presented by the Institute of Acarology, Ohio State University.

Invited to contribute a chapter on microenvironment of desert rodent burrows for a book on rodents in desert environments.

Reviewed grant proposals for the Tropical Medicine and Hygiene Study Section, Division of Research Grants, and research manuscripts for the Journal of Parasitology, Experimental Parasitology and Journal of Medical Entomology.

Continued to serve as a member of the Editorial Board of the Journal of Medical Entomology.

Invited to teach a portion of the course "Invertebrate Cell Culture" at the W. Alton Jones Cell Science Center, Lake Placid, N.Y.

Appointed as Affiliate Professor of Zoology in the University of Montana.

Dr. R. Parker

Invited to participate in a Symposium on Significance of Insect and Mite Nutrition, University of Kentucky, Lexington.

Reviewed research manuscripts for Journal of Parasitology and Journal of Medical Entomology.

Publications:

Cory, J. and Yunker, C. E.: Primary cultures of tick hemocytes as systems for arbovirus growth. Ann. Entom. Soc. Amer. 64: 1249-1254, 1971.

Yunker, C. E.: Arthropod tissue culture in the study of arboviruses and rickettsiae: A review. In Weiss, E. (Ed.): Current Topics in Microbiology and Immunology 55. New York, Heidelberg, Berlin, Springer-Verlag, 1971, pp. 113-125.

Cory, J. and Yunker, C. E.: Arbovirus plaques in mosquito cell monolayers. Acta Virol. 16: 90, 1972.

In press:

Yunker, C. E. and Cory, J.: Growth of Rickettsia tsutsugamushi in insect cells. In Proc. 3rd Int. Coll. on Inv. Tissue Culture, Smolenice, 1971.

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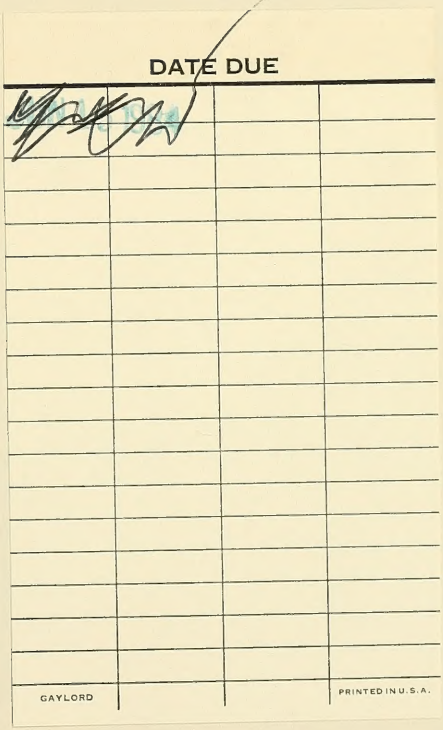

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